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Renewable Energy System by Biological Solar Energy Conversion

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BIOHYDROGEN III

Renewable Energy System by Biological Solar Energy Conversion

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PREFACE

Hydrogen is regarded as one of the most promising energy carriers of our future: This is especially due to the fact that it can be regenerated in a cyclic process out of water without emission of CO_2, i.e. it is environmentally neutral.

The main problem is that hydrogen gas does not exist as a pure compound in natural resources. For this reason it has to be produced by technical processes from fossil energy carriers which in turn usually require high temperatures and high pressure. In addition, the production of the unwanted CO_2 is inevitably involved in these processes. Hydrogen can also be technically produced from water by electrolysis using conventional or regenerative produced electrical energy. However, as the efficiency of this process is rather low (about 10%) it is quite expensive. An alternative, CO_2 neutral method is the photobiological hydrogen production by microalgae which use natural solar energy directly as energy source for these transformation processes. These organisms whose growth rates are about 10-times higher than those of higher plants grow with minimal nutrients due to a very efficient photosynthesis. Some of them contain hydrogenases with an extreme capacity for the production of hydrogen. In contrast to technical processes, photobiological hydrogen production does not require high-tech equipment as all processes occur at room temperature and at atmospheric pressure. Moreover, as no electricity has to be generated transiently, the transformation efficiency is rather high – usually more than 10%. Biohydrogen is pure hydrogen, so there is no need for further purification processes and conclusively no air pollution occurs.

The use of such natural hydrogen production machines in combination with the natural process of photosynthesis is the topic of an international NEDO project for the development of a semiartificial device for hydrogen production. On the occasion of the second meeting of all groups involved in this project, an international symposium on "Biohydrogen" was organized in Kyoto 2002. The *state of the art* of biohydrogen production from participants of this symposium is summarized in the chapters of this book.

October 2002

NEDO International Joint Research Grant
"Research team of Molecular Device for Hydrogen Production"
Team Leader, Matthias Rögner

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I. Hydrogen Production

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NEW FRONTIERS OF HYDROGEN ENERGY SYSTEMS

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ABSTRACT

The developments of the proton exchange membrane fuel cells (PEMFC) and PEMFC-applied compact engines, for recent several years, have given rise to the breakthrough of the hydrogen utilization systems. On the other hand, the C-nanostructures for hydrogen storage systems for vehicles has been regarding as the ace of frontiers, but it is, as yet, not quite satisfactory. The on site cogeneration systems of pipelines combined with the fuel cells will be realized in near future. It is surely expected that the hydrogen supply shortage will occur in 21 Century, so that the emerging frontiers will be the hydrogen production technologies from water such as solid polymer water electrolysis, biolysis applied by the genetic study, mechanolysis, and sono-fusion, which is an extension of water sonolysis of water. It is to be hoped that the all energy resources will be met by renewable energies.

INTRODUCTION

The spurred impetus has been given to developing non pollutant vehicles, and consequently, the clean cars driven by the fuel cells loading proton exchange membranes (PEMFC), which based upon Nafion, have been surprisingly developed. A promising less pollutant and economical system is also expected, which will be the on site cogeneration system of electric power and the hot water supply with use of fuel cells combined with city gas pipe-lines.

Such a spreading trend of non pollutant systems by hydrogen utilization would yield shortage of hydrogen supply in 21 Century Accordingly, new frontiers of hydrogen energy systems will be the hydrogen production systems using renewable energy resources. In this concern, it should be noticed that the nuclear emissions due to D-D collisions were observed by the strong implosion of cavitation bubble in the acetone pool in a beaker [1]. Most of the responses so far are negative [2], however a faint possibility is also reported [3-5]. If it is true, the ultimate hydrogen energy systems will be furnished. An introduction of this "bubble fusion" is an unique part of this paper, which is not published before [6].

The technologies for hydrogen storage will shoulder the center of hydrogen systems, and they have been in keen competition with each other. However, the DOE's object for innovative technologies is far beyond the reach, presently. The carbon nano structures for

hydrogen storage have been actively investigated, but no goal is foreseen yet.

The principles for hydrogen utilization systems are also discussed, and the frontier examples are introduced.

In each area of hydrogen energy systems, biohydrogen technologies will play the important roles, because they are the traditional, effective, and safe conversion and storage methods of solar energy. It should be emphasized that the application of genetics is so unique that no other technologies can compete with biohydrogen technologies..

Figure 1. A typical hydrogen car developed by BMW. by the side of a wind mill field.

WATER-SPLITTING SYSTEMS BY RENEWABLE ENERGY

Presently, more than 98 % of the hydrogen gas consumed by the industries are provided by reforming coal, naphtha, and natural gas, and will be unable to bear the future demand [7]. It is strongly required to supply the hydrogen produced from water by renewable energy sources.

Table 1 shows the water-splitting methods (-lysis) by the different kinds of energies. Hydrogen produced by water electrolysis is the traditional way since M. Faraday, however it cannot be qualified as clean energy carrier because of its energy resources, unless the electric power is generated by renewable energy. Accordingly electrolysis should be combined with for instance, solar cell, solar thermal, etc.

It is noticed that the improvement of solar cells is remarkable, and the efficiencies of Si-single crystal cell and the poly crystalline cell reach 17 % and 12.5 %, respectively. The average cost of solar cell module is $3 per watt, which can be competitive with other conventional power sources [8].

Author has introduced the discovery of mechanolysis, a novel phenomenon of water splitting [9,10], which has been understood as a result of frictional electricity between the Teflon stirring rod and the Pyrex glass of the beaker, where pure water containing semiconductor powder is filled. Author [9] has pointed opt that the semiconductor must have the property of the hopping conductivity, and called tribolysis. There exists another type of mechanolysis, which may be due to the piezo electrolysis. This type is called piezolysis, but not discovered yet.

However, a giant piezoelectric effect has been found in the Pb-based complex pervoskite oxides. In particular, the morphotropic boundary relaxor and $PbTio_3$ complex exhibits huge piezoelectric response, so that an effective piezolysis is expected.

Another big merit of mechanolysis system combined with wind power, relative to wind

electric generation, is that hydrogen can be stored in a vessel.

Water vapor is split into its components at the temperatures higher than ~4200 k, then hydrogen will be given by separating the mixture gas. This method is called direct thermal water-splitting. As the high temperature technologies are so difficult that this may not be promising. However, thermal energy is useful to split water especially in thermochemical method [11] and in fermentation [12]. Fermentation does not need very high temperature and is environmentally friendly, and is expected to be one of the aces of the frontiers.

Water-splitting by thermal energy is called pyrolysis.

The global surface is filled of sunshine, total amount of which is more than ten thousand times compared to the total consuming energies by mankind.

Table 1. Water-splitting methods by renewable energies

( ) means duplicate. *Piezolysis is not discovered yet.
*** Bubble fusion is not confirmed yet, which is a continuance of water-sonolysis**.

1. Electrolysis	(1) combined with renewable power systems[a)]
2. Mechanolysis	(1) tribolysis, piezolysis*
	(2) sonolysis**
3. Pyrolysis	(1) thermochmical[b)]
	(2) direct thermal
	(3) fermentation[c)]
4. Photolysis	(1) photoelectrochemical
	(2) photobiochemical
	(3)(solar cell combined with electrolysis)[a)]
5. Chemolysis	(1) (density gradient combined with electrolysis)[a)]
	(2) (ion exchange membrane)[a)]
	(3) (thermochemical)[b)]
6. Biolysis	(1) living systems
	(2) cell free systems
	(3) (fermentation)[c)]

[7]Bubble fusion: D-D fusion triggered by the implosion of cavitation bubble.***

Solar energy with the short wave length range and long wave range can be utilized by photolysis and by pyrolysis, respectively. As for photolysis, we have (1) the biological area based upon the photosynthesis, and (2) the electrochemical area such as photoelectrochemical with photo semiconductor, with dye and metal complex etc. [13].

Photoelectrochemical water-splitting is a combination of solar cell with electrolysis in a electrolyte , and has been actively studied. However, the selection of the photo semiconductors is so tightly limited that photoelectrochemical methods can hardly compete with the combined system of solar cell with electrolysis.

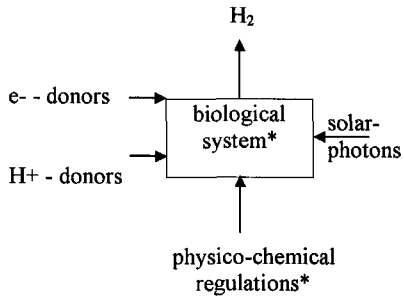

Figure 2. Hydrogen production model by living systems. *
: Including the genetic applications.

On the other hand, as is repeated so far, the biolysis has a bright future because of the biological system, which may be improved by the genetic evolution (Fig. 2 [14]).

Besides the subjects in Table 1, someone would list up radiolysis, which is the water-splitting system by radioactive rays. However, it belongs to a kind of photolysis, and has apprehensions that the produced hydrogen may carry the contaminated radioactivity.

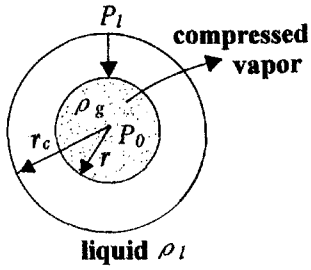

Figure 3. Bubble collapse. ρ_g and ρ_l represent the vapor and liquid density, respectively.

SONOLYSIS AND THE BUBBLE FUSION

It is possible to split water by irradiating ultra sound wave (USW) with 50 - 300 [kHz] onto water [15]. This phenomenon is called water sonolysis. If the cavitation bubble in water expands to the size with radius r_c ($\approx 10^{-4}$ m), and then implodes to a smaller bubble with radius r_0 ($\approx 10^{-6}$ m), the temperature inside the smaller bubble will rise to T_0 given by

$$T_0 = \left(\frac{r_c}{r_0}\right)^3 T_C \tag{1}$$

where k is Boltzmann constant and T_C is the critical temperature of water.

Eq. (1) shows that T_0 can be higher than 10^8 K (even near 10^9 K), so that water vapor is split into its components to yields sonolysis.

The energy balance equation is given by

$$4\pi\gamma r_C^2 = \frac{3}{2}NkT_0 + N\varepsilon \tag{2}$$

whereγ, N, ε, and k are the surface tension, the number of the elements, the separation energy, and Boltzmann constant, respectively. The r.h.s of Eq. (2) is responsible to direct pyrolysis.

In the spring of 2002, the research group of Oak Ridge National Laboratory [ORNL] has reported [1] that if USW is irradiated on deuterated acetone (C_3D_6O), nuclear emission is observed and the thermonuclear reactions:

$$D^+ + D^+ = T^+ + H^+ + 3.26\,\text{MeV} \tag{3}$$

$$D^+ + D^+ = {}_3He^{2+} + n + 3.26\,\text{MeV} \tag{4}$$

may occur.

Author has studied the phenomenon in detail, and published the results [3-5] that the observable possibility is appreciable, while Lawson condition is not satisfied. In order to realize the nuclear emission, both the plasma temperature (T_0) and the density of D ions (n_D) should be large enough to satisfy the required conditions. The density n_D is determined by plasma density, which depends upon the vapor pressure in the initial bubble.

The thermal energy generated by the release and the concentration of the molecular binding energies of the pool materials is consumed partly to manufacture the plasma, and partly to rise up the temperature. If the vapor pressure is too high, no nuclear emission will occur, because the energy is not enough to ionize too many elements.

Figure 4 is the energy flow diagram from the molecular system to the nuclear reaction system, and the key properties of the pool materials and the key parameters of the system are shown.

Illinois group [2] has expressed a negative version on ORNL group, but their estimation of r_C was done for water, so that it was too small to give enough potential to the initial bubble. Their version cannot be applied to deuterated acetone.

Author has studied the key properties of the pool materials and the effective conditions of USW absorption, and he believes that bubble fusion will be one of the frontiers of hydrogen energy systems in 21 Century.

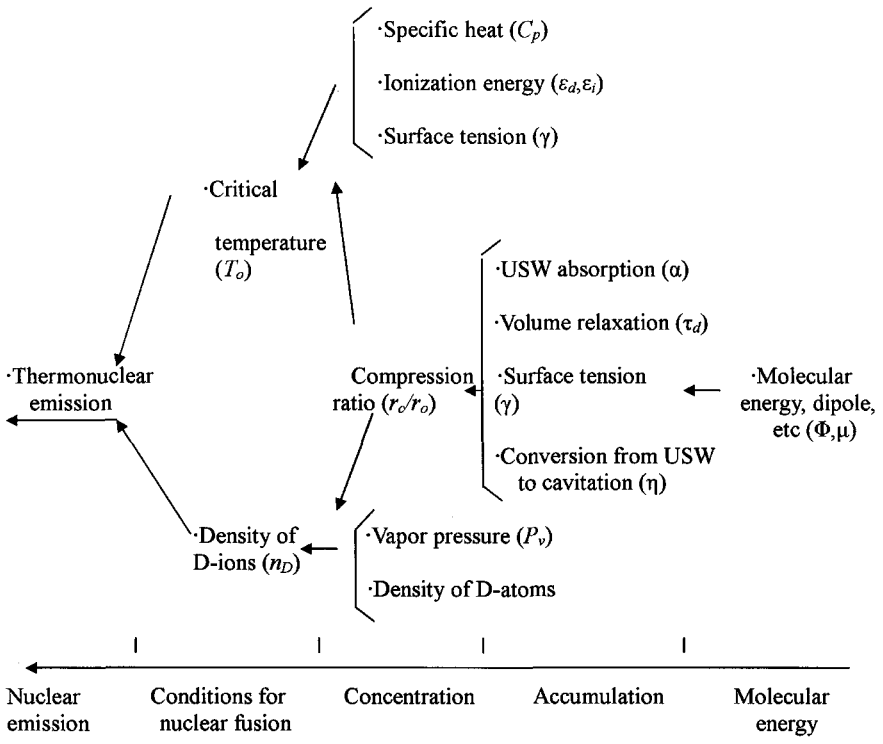

Figure 4. Energy flow diagram and the properties of pool material [5].

HYDROGEN STORAGE SYSTEMS

As the hydrogen fueled cars have been developed so fast, the safe and effective storage technologies for hydrogen have been greatly interested. Pressure steel vessels and liquefaction are the traditional ways, however, the former is heavy, and the latter is expensive not only for the apparatuses but also for the liquefaction, and they are not neccessarily fit for the car. Department of Energy (DOE) in USA has shown the target for the effective hydrogen storage, which is shown in Figure 5. [16].

Let's briefly review the promising hydrogen storage methods by Figure 5, where the volume densities (V_d) vs weight densities (W_d) for each storage method are shown [16].

Pressurized hydrogen vessels made of steel is too weighty to carry with cars, and liquefied hydrogen cryogenic method has no infrastructures yet. Metalhydrides is meritorious for V_d, but not for W_d.

Single walled carbon nanotubes (SWNT) methods are most promising and its V_d is more than 50 [kg/m^3] at the highest, and its W_d is about 5 wt.%. Recently, Cal. Tech. group has found that W_d can be 8.25wt. % at 80 K and under the pressure12MPa. N. M. Rodriguez and P. E. Anderson [17] have reported that graphite nanofibers may store hydrogen with

W_d = 68 wt. %, which is extraordinary, however, it is reported that many pursuits could not reconfirm.

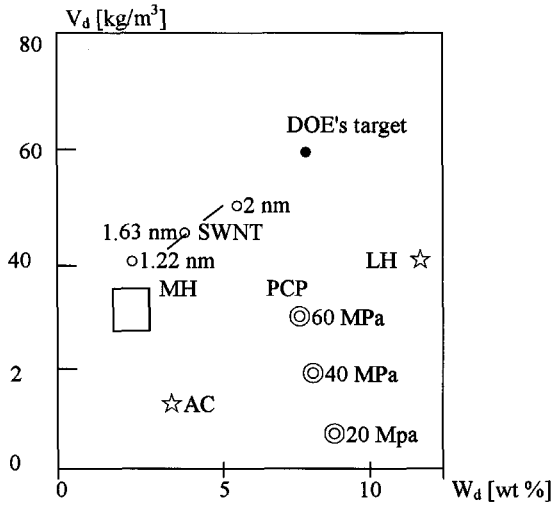

Figure 5. H_2-storage by different methods.
V_d, W_d, MH, SWNT, PCP, and AC mean volume density, weight density,metalhydrides, single walled carbon-nanotubes, pressurized carbon polymer, and activated carbon, respectively.

On the other hand, hydrogen storage by pressurized carbon polymers (PCP) is effective in W_d, of which efficiency is much higher than that of the activated carbon.

It is concluded that SWNT will be most promising. However, the storage mechanism is not clarified yet, i.e., whether the absorption is due to the physical reaction or the chemical reaction. Nevertheless, it is not clarified yet whether only the inside of SWNT is responsible to the absorption or not. The out side plays also the role, in some cases, may be.

We must notify that gasoline, methanol, and LPG are also the storage methods if the reforming apparatus are provided. Biomass also may be applied.

HYDROGEN UTILIZATION SYSTEMS

The precious ways of hydrogen utilization have the principles based upon the two non substitutive properties of hydrogen, that is, hydrogen energy systems are not only ecological but also energetic.

Energetic means that hydrogen combustion has the high power (chemical wattage) that generates a big energy per unit time, which has been applied to the second stage of rocket launching.

Ecological means that hydrogen can make not only the on site recycle by reversible

physical reaction and/or by chemical reaction cycle but also the global recycle by irreversible consumption via water-generation and its splitting.

The utilization principles are shown in Figure 6, where the typical examples are enumerated. Hydrogen turbine has been studied by Japanese WE-NET project and the achieved energy efficiency was as high as about 60 %, which can be competitive with fuel cell system. One of the typical direct energy conversion systems, which have no movable parts and no noise, is fuel cell. Today' topics of clean cars have been focused to the cars with PEMFC as was mentioned previously.

Here, we should notice that production of organic matters from carbon dioxide using hydrogenotrophs may play an important role in future [18].

One of the local recycle systems (LRS) of hydrogen utilization is due to physical and reversible reaction as [19]

$$2M + H_2 \leftrightarrow 2MH + \Delta q, \tag{5}$$

where M and Δq mean the alloy and the reaction heat, respectively. This utilization system is called metalhydride system (MHS). The development of M is essential to MHS.

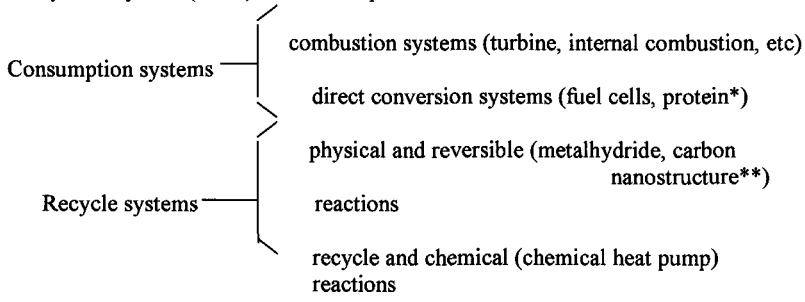

Figure 6. Utilization principles of hydrogen.
* Synthesis of hydrogen-protein will be an emerging object.
** Hydrogen absorption by carbon nanostructure is not always due to the physical reaction.

Another LRS is the chemical and recycle reaction system as [20]

$$\begin{aligned} &C_3H_8O \rightarrow C_3H_6O + H_2 \\ &C_3H_6O \rightarrow C_3H_6\,O \quad \text{(gasfication)} \\ &C_3H_6O + H_2 \rightarrow C_3H_8O \\ &C_3H_8O \rightarrow C_3H_8O \quad \text{(liquefaction)} \end{aligned} \tag{6}$$

can be applied as an effective chemical heat pump, where no hydrogen is consumed. Eq. (4) is the reversible cycle between 2-propanol and acetone, which can take place below 100°C, and will come into wide use in near future.

SUMMARY

The new frontiers of hydrogen energy systems described in this paper will be PEM-electrolysis combined with renewable energy sources, biolysis with use of biological methods based on the genetics, and mechanolysis combined with any moving phenomenon and object, in hydrogen production area.

The special and dreamful subject is the bubble fusion, which must be thoroughly investigated, and if we can find the evidences, an evolutional energy system will be organized.

SWNT is the ace of frontiers for hydrogen storage systems, but biological methods can be the rival, if ad hoc genetics is applied.

PEM fuel cells and chemical heat pump will be the new frontiers in the hydrogen utilization systems. However, if hydrogen protein can be biologically created, it will be a great gospel for mankind.

Lastly, let us close by citing J. Refkin's phrase, "*The creation of the world-wide energy web and the redistribution of power on earth.*" [21].

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NOVEL APPROACHES TO EXPLOIT MICROBIAL HYDROGEN METABOLISM

Kornél L. Kovács, Z. Bagi , B. Bálint , B. D. Fodor , Gy. Csanádi, R. Csáki, T. Hanczár, Á. T. Kovács, G. Maróti, K. Perei, A. Tóth and G. Rákhely

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ABSTRACT

The purple sulfur phototrophic bacterium, *Thiocapsa roseopersicina* BBS contains several [NiFe] hydrogenases. Two membrane bound [NiFe] hydrogenases were characterized. One of these enzymes (HynSL) is remarkably stable and can be used e.g., as fuel cell H_2 splitting catalyst. A third hydrogenase activity was located in the cytoplasm and was analogous to the NAD-reducing hydrogenases. In addition, the genes homologous to the hydrogen sensing hydrogenase have been sequenced. Although all elements of a typical H_2 sensor (*hupUV*) and two-component regulator (*hupR*, *hupT*) are present, they appear to be non-functional. The synthesis of HydSL/HynSL protein seems to be redox regulated.

Some of the accessory genes were identified using random mutagenezis. One of the mutations was in the gene coding for the HypF proteins. Inactivation of [NiFe] hydrogenase biosynthesis in the *hypF* deficient mutant resulted in a 60-fold increase in hydrogen evolution capacity of *T. roseopersicina* under nitrogen fixing conditions. In a distinct mutant the inactivation of the *hupK* gene yielded a nitrogenase independent photoheterotrophic H_2 production.

Methanotrophic bacteria utilize H_2 to supply reductant for their methane monooxygenase (MMO) enzyme systems. H_2 driven enzyme activity plays determining role in methane oxidation. This process is of great importance in decreasing the emission of the greenhouse gas methane, in bioremediation of halogenated hydrocarbons and related hazardous compounds, and in formation of the easily storable and transportable renewable energy carrier methanol. [NiFe] hydrogenases participating in the related biochemical events were identified and studied from the moderately thermophilic *Methylococcus capsulatus* (Bath).

Microorganisms that supply H_2 in situ facilitate the biodegradation of organic material and concomitant biogas production. Fast, efficient, and economic treatment of organic waste, sludge, manure is achieved and generation of significant amount of renewable fuel from waste is intensified. The technology has been field tested under mesophilic and thermophilic conditions with positive results.

HYDROGENASES

Understanding the molecular fundamentals of hydrogen production and utilisation in biological systems is a goal of supreme importance for basic and applied research [Cammack et al., 2001]. The key enzyme in biological H_2 metabolism is hydrogenase. This unique enzyme catalyses the formation and decomposition of the simplest molecule occurring in biology: H_2.

$$H_2 \rightarrow 2H^+ + 2e^- \qquad 2H^+ + 2e^- \rightarrow H_2$$

It should be noted that hydrogenases can help us in two ways: they may catalyse both H_2 generation (e.g., photobiological or fermentative) and H_2 consumption (e.g., in fuel cells).

This simple-looking task is solved by sophisticated macromolecular machinery. Hydrogenases are metalloenzymes harbouring Ni and Fe, or only Fe atoms, arranged in an exceptional structure. This study focuses on the hydrogenases with NiFe active centres. Like most redox metalloenzymes, hydrogenases are usually extremely sensitive to inactivation by oxygen, high temperature, CO, CN and various environmental factors. These properties are not favourable for most biotechnological applications, including biohydrogen production, water denitrification, bioconversion of biomass, and other bioremediation uses.

Hydrogenases are found in Archaea, Eubacteria and simple Eukaryota. Their physiological function vary: they can serve as redox safety valves to dispose of excess reducing power, or generators of chemical energy by taking up and oxidising H_2, or maintaining a reducing environment for reactions of crucial importance, such as the fixation of atmospheric nitrogen. In some organisms, the numerous functions are performed by the same enzyme, but more frequently, a separate, specialised hydrogenase carries out each *in vivo* biochemical function.

Hydrogenase structure

In metal-containing biological catalysts, it is the protein matrix, surrounding the metal centres, which provides the unique environment for the Fe and Ni atoms and allows hydrogenases to function properly, selectively, and effectively. Hydrogenases are ancient enzymes, hence their protein matrix is rather conserved. The NiFe hydrogenases are composed of at least two distinct (heterodimer) polypeptides, containing highly conserved metal binding domains. The large subunit harbours the active centre, fastened to the protein by 4 cysteine ligands. The Fe atom ligates 2 CN and 1 CO diatomic molecules and it is fixed to the Ni atom via sulphur bridges (Fig. 1). Similar heterobinuclear NiFe centres are not known in any other metalloenzyme. The presence, the incorporation mechanism, and the function of the CN and CO groups are mysterious as both cyanide and carbon monoxide are poisonous for the micro-organisms and irreversibly inactivate the NiFe hydrogenases themselves when administered externally. The small subunit contains 2-3 Fe_4S_4 clusters, which are precisely and equally spaced, 15 angstroms apart, and thus, form a conducting wire inside the protein to facilitate the transport of electrons between the active centre and the protein surface. A major goal for hydrogenase basic research is to understand the intimate protein-metal interaction in this complex structure [Cammack et al., 2001].

The problem is not simple to address, as some of the methods for scientific investigation provide information on the metal atoms, without directly detecting the protein matrix around them. Other modern techniques reveal details of the protein core, but do not expose the metal centres within. A combination of the various molecular approaches is expected to uncover the fine molecular details of the catalytic action [Kovács and Bagyinka, 1990, Szilágyi et al., 2002]

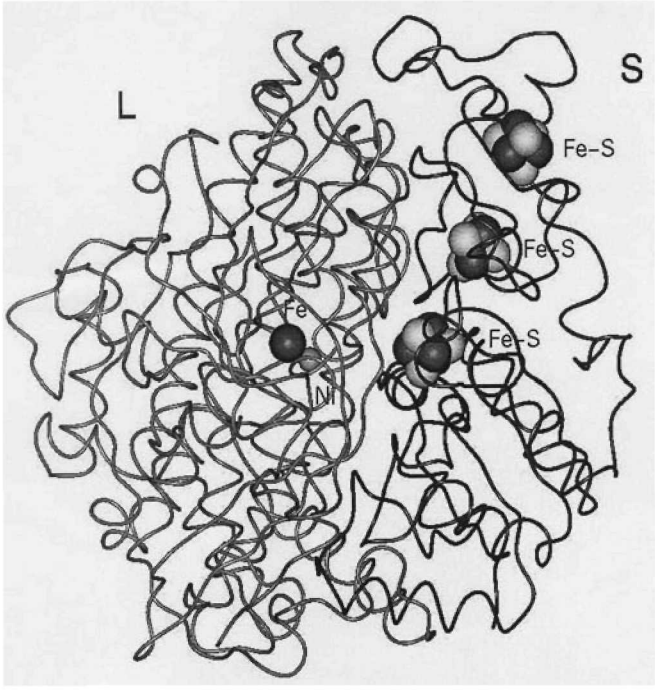

Figure 1. The structure of NiFe hydrogenases. The large subunit polypeptide (backbone is indicated in grey) harbours the unique NiFe active centre, the small subunit polypeptide (backbone is indicated in dark grey) contains the Fe_4S_4 clusters for the transfer of electrons between the protein surface and the NiFe centre.

Assembly of NiFe hydrogenases

In order to develop suitable biocatalysts for future biotechnological applications, the structure-function relationship, biosynthesis and assembly of hydrogenases must be understood. Determination of the protein primary sequences from the structural genes is clearly necessary, but not sufficient, requirement. A number of other gene-products govern the metal uptake, their attachment into the right place at the right time, formation and ligation of the CN and CO groups, and the incorporation and fixation of this labile inorganic structure into the protein matrix. Our present understanding suggests that the concerted action of, at least, 15-20 such accessory proteins is necessary for the formation of an active NiFe hydrogenase [Cammack et al., 2001]. This well organised "assembly-line" works in the nanoscale range in both space and time. Consequently, in a millilitre of bacterium culture, several million identical assembly lines operate, each having the complexity of a car factory. Some of the participating proteins are *hy*drogenase *p*leiotrop, called Hyp. They take part in the fabrication of every hydrogenase synthesised in the cell. Others specifically work on one type of NiFe enzyme and therefore several variants of the similar accessory proteins may exist in the same micro-organism.

An almost uniform organisational scheme is observable for the structural genes: the gene coding for the small subunit precedes the one coding for the large subunit and the two genes form one transcriptional unit. Sometimes, the accessory genes are neatly arranged around the structural genes, but most often, they are scattered in the genome.

Photosynthetic bacteria

The best sources of hydrogenases, both for basic research and for forthcoming large-scale utilisation, should be micro-organisms that are cheap to cultivate and use sunlight to get energy for their growth. A group of likely candidates are phototrophic bacteria: they carry out anaerobic photosynthesis via PhotosystemI and do not contain the oxygen producing PhotosystemII present in higher photosynthetic organisms, such as algae and green plants [Sasikala et al., 1993]. Consequently, phototrophic bacteria do not generate oxygen during growth, which could inhibit the biosynthesis and/or activity of hydrogenases. Another property of anaerobic photosynthesis is the requirement of suitable electron donor(s) to feed electrons into the photosynthetic electron transport chain [Kovács et al., 2000]. Many phototrophs use sulphide (or other reduced sulphur compounds) as electron source, which prevents the accumulation of poisonous sulphide in the environment. One such phototrophic bacteria is our favourite organism, *Thiocapsa roseopersicina*.

Why Thiocapsa roseopersicina BBS?

T. roseopersicina is a phototrophic purple sulphur bacterium; the strain marked BBS has been isolated from the cold water of the North Sea. Its anaerobic photosynthesis uses reduced sulphur compounds (sulphide, thiosulfide, or elementary sulphur), but it can also grow on organic compounds (sugar, acetate) in the dark. The bacterium contains a nitrogenase enzyme complex, thus it is capable of fixing atmospheric N_2, a process accompanied by H_2 production [Vignais et al., 1995].

Previous studies in our laboratory have revealed that *T. roseopersicina* contains at least two membrane-associated NiFe hydrogenases with remarkable similarities and differences. One of them (HydSL/HynSL [for recent nomenclature change see Vignais et al., 2001]) shows extraordinary stability: it is much more active at 80°C, than around 25-28°C. It is to be

noted that *T. roseopersicina* cannot grow above 30°C. HynSL of *T. roseopersicina* is also reasonably resistant to oxygen inactivation and stays active after removal from the membrane. The other NiFe hydrogenase, HupSL, is very sensitive to all these environmental factors and thus it resembles the NiFe hydrogenases known from other micro-organisms. The structural genes coding for these enzymes have been cloned and sequenced [Colbeau et al., 1994; Rákhely et al., 1998]. The translated protein sequences indicate a significant sequence homology between the two NiFe hydrogenases. Despite the pronounced differences in stability, the two small subunits are identical in 46% of their amino acids and the two large subunits show 58% sequence identity. In order to understand the physiological roles of these hydrogenases, mutants lacking either or both of them have been generated in our laboratory by marker exchange mutagenesis. Much to our surprise, the hydrogenase-deleted mutants grew just as avidly as the wild type strain. The phenomenon was finally understood when two additional NiFe hydrogenases were discovered in the cytoplasm of the bacterium. According to our current understanding, there are four distinct NiFe hydrogenase molecular species in *T. roseopersicina*, representing all hydrogenase forms thus far described in various micro-organisms, in a single cell [Kovács et al., 2002]. This makes *T. roseopersicina* one of the best candidates for studies of NiFe hydrogenase structure-function relationships and assembly. The outstanding situation allows us to address specific questions concerning the assembly of each of these enzymes and the regulation of their biosynthesis. Answers to these questions will be of direct relevance in designing an optimal catalyst for biological hydrogen production and/or utilisation and to protein engineering of scientifically intriguing and biotechnologically important redox enzymes in general. In the following section the characteristics of the four NiFe hydrogenases (Fig. 2) will be summarized.

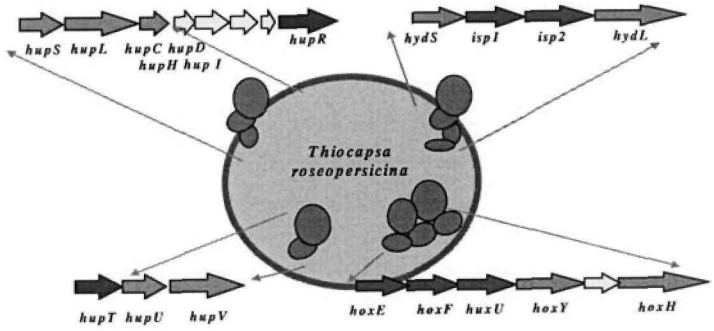

Figure 2. Hydrogenases in *T. roseopersicina* and the organisation of the gene clusters coding for the corresponding proteins.
The locationof the enzyme macromolecules (grey) is indicated schematically within the cells of this purple sulfur bacterium (purple). The structural genes are marked in red, genes coding for proteinsthat form a stable complex with the hydrogenase subunits are coloured green, genes coding for accessory proteins are yellow, regulatory genes are blue and genes coding for proteins of unknown function are light blue.

Stable hydrogenase (HydSL/HynSL):

Commonly called Hyd, a recent nomenclature revision proposes a new abbreviation of Hyn for these enzymes [Vignais et al., 2001]. In addition to its outstanding stability, this enzyme is noted also for the unusual organisation of the structural genes coding for the Hyn-hydrogenase. Unlike most hydrogenase structural gene clusters, the gene coding for the small subunit, *hynS*, is separated from *hynL* with an approximately 2 kb long DNA segment, containing genes for two putative proteins. The role of this arrangement and the properties of the putative proteins are subjects of ongoing research in our lab. HynSL has been purified as an active hydrogenase heterodimer to homogeneity [Kovács et al., 1991]. The purified enzyme is stable, similarly to the membrane associated *in vivo* state. *In vitro* HynSL catalyses both H_2-evolution and H_2-uptake, but it functions primarily in H_2 consumption in the living bacterium.

Unstable hydrogenase (HupSL):

HupSL also functions in the H_2-uptake direction *in vivo*. Its sequence shows high homology to HynSL, but it is so unstable that we have not yet been able to isolate the protein from the membrane in an active form. A comparative study with HynSL, at both molecular biology and protein biochemistry level, will hopefully shed light on the structural basis of the stability differences [Szilágyi et al., 2002]. NiFe hydrogenases related to HupSL have been found and studied in a number of micro-organisms. In other systems, biosynthesis of HupSL is linked to the nitrogen fixation process and the generally assumed physiological role of HupSL is to recycle the excess H_2 produced by the nitrogenase enzyme complex [Colbeau et al., 1994; Cammack et al., 2001]. Very interestingly, in *T. roseopersicina* the translation of *hupSL* is apparently unrelated to nitrogen fixation and H_2 does not regulate how many copies of this enzyme are present in the cell, but the HupSL activity is H_2 regulated (Á. T. Kovács et al., unpublished results).

Soluble hydrogenase (HoxYH):

This is one of the cytoplasmic hydrogenases discovered recently in *T. roseopersicina*. In fact the structural gene cluster predicts that it is a five subunit enzyme, coded by the *hoxEFUYH* gene cluster. The *hox* gene products are related to HoxFUYH, described in detail in *Ralstonia eutropha*, a chemolithotrophic bacterium [Friedrich and Schwarz, 1993]. The corresponding structural genes have been sequenced, the purification and characterisation of this hydrogenase from *T. roseopersicina* is in progress. The soluble hydrogenase functions primarily in the direction of H_2 production, therefore its study should give us information, which will be useful in designing biocatalysts for biohydrogen production.

Sensor hydrogenase (HupUV):

A set of genes homologous to *hupTUV/hoxJBC* in *Rhodobacter capsulatus* and *R. eutropha*, respectively, has been identified and sequenced from *T. roseopersicina*. HupUV senses H_2 in the environment of *R. capsulatus* and triggers the biosynthesis of the only hydrogen-uptake enzyme, HupSL in this organism [Elsen et al., 1996]. A similar function has been assigned to the homologous HoxBC protein in *R. eutropha* [Friedrich and Schwarz, 1993]. Interestingly, external H_2 signals do not regulate the transcription of none of the NiFe hydrogenases in *T. roseopersicina*. Therefore, a H_2 sensing hydrogenase has regulatory function at posttranscriptional level.

From the fragmented information available, there is no clear answer as to why

T. roseopersicina needs so many distinct hydrogenases. Our working hypothesis links this abundance of various NiFe hydrogenases to the fact that this bacterium should be able to perform various metabolic activities (photoautotrophic, photoheterotrophic, heterotrophic metabolism) in order to survive in its natural habitat [Imhoff, 2001]). Having numerous hydrogenases at hand increases the chances of survival for the bacterium and increases our chances to understand basic phenomena of hydrogenase catalysis.

Accessory genes participating in the assembly of NiFe hydrogenases in T. roseopersicina

The advantages of having at least 4 types of NiFe hydrogenases in *T. roseopersicina* are counter balanced by the fact that most of the accessory genes needed for their assembly and biosynthesis are scattered in the genome. The downstream region of the *hupSL* structural gene cluster contains some of these genes: *hupCDHI* and *hupR* have been identified on the basis of their sequence homology to the corresponding accessory genes in other micro-organisms [Colbeau et al., 1994]. No accessory genes have been found around the other three structural gene clusters. This situation is not unique among the micro-organisms, but due to the numerous NiFe enzymes present in *T. roseopersicina*, a large number of accessory genes must be found and characterised in order to locate the elements of the hydrogenase "assembly-line". Since some of these genes are pleiotropic, the number of missing genes is estimated to be between 20 and 30. The majority of these genes and their protein products are needed for the macromolecular structure-function studies; therefore a systematic search has been launched in our research team using two approaches. We have begun sequencing the genome of *T. roseopersicina*, which is a laborious and expensive approach and it will produce more sequence information than required for solving this particular problem. At any rate, the information necessary for the identification of all accessory genes will be included in the database, when completed. Random mutagenesis and screening for altered hydrogenase phenotypes allows us to identify those genes that play a significant role in the formation of the functionally intact enzymes. This is a straightforward approach, so long as there is a good method available to screen the mutants and the mutation causes phenotypic change(s). It should be noted that the two approaches provide complementary information and their simultaneous application is therefore justified. The goal of this work was to identify hydrogenase accessory genes in *T. roseopersicina* by random transposon mutagenesis.

First, genetic tools appropriate for use in *T. roseopersicina* had to be developed since no molecular genetic work had been done on this micro-organism. An efficient conjugative gene transfer system was employed [Fodor et al., 2001]. Second, a random transposon mutagenesis system was adopted using a wide host range plasmid and Tn5 transposon derivative (Fig. 3). Third, an efficient screening method was necessary to detect the mutants with altered hydrogenase activity within a large mutant library. The H_2-uptake activity of colonies was observed using the selective and indicative colour change of the redox dye, methyl viologen (Fig. 4).

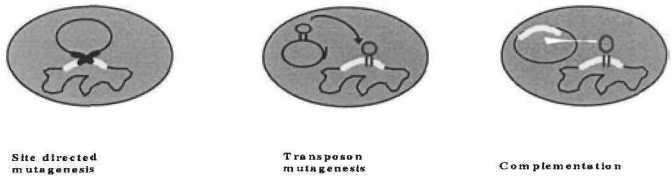

Figure 3. Genetic tools developed for *T. roseopersicina*.
Tn5 based transposonmutagenesis combined with complementation experiments were used in search of the hydrogenase accessory genes. The target gene is markedwith yellow, transposon is blue and the shuttle vector is indicated with a circle. The vector delivering the transposon will be eliminated after the random insertion of the transposable element. Insertion of the transposon inactivates the target gene function; this can be corrected via introduction of the gene on a vector (complementation).

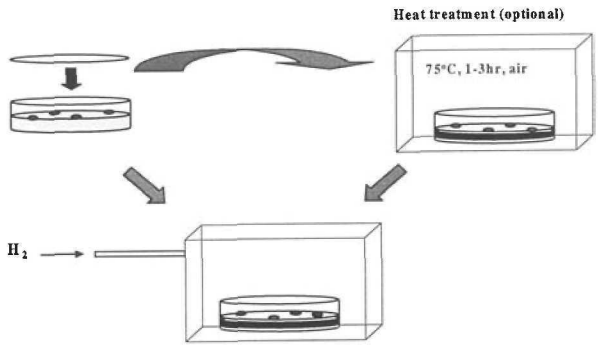

Figure 4. Screening for hydrogenase deficient phenotype.
The purple colonies of *T. roseopersicina* are lifted on a filter paper, transferred onto a stack of filter papers soaked with oxidised redox dye (benzyl viologen) under air. Following heat treatment, the cells containing heat stable, active enzyme turn blue under hydrogen atmosphere,those containing defected hydrogenase remain purple [Fodor et al., 2001].

INVENTORY OF THE IDENTIFIED ACCESSORY GENES

As mentioned above, the genes *hupCDHI* and *hupR* were previously identified in the *hup* gene cluster earlier [Colbeau et al., 1994]. These will not be discussed in detail here.

hypC

The *hypC* gene codes for a small protein, which plays a chaperone role during the assembly of one of the NiFe hydrogenases in *Escherichia coli* [Drapal and Böck, 1998]. The homologous protein has been found in several other bacteria too and they have been assigned a similar function. The gene product, *hypC*, binds to the nascent large subunit polypeptide and holds it in the proper "open" conformation while other proteins insert the metal centres. Although it is described as having a pleiotropic effect, we have identified two very similar but distinct *hypC* genes in *T. roseopersicina*.

hypD

Most likely, this gene also codes for a pleiotropic accessory protein. In practically all micro-organisms, where *hypD* has been found, it is always clustered with *hypC*. There is no explanation for this salient phenomenon. In our case the two genes overlap in a three base-pair length, which is a sign of strong coupling between them. The *hypCD* cluster was the first set of accessory genes identified in hyperthermophilic Archaea [Takacs et al., 2001] indicating that the hydrogenase "assembly-line" has been conserved through huge evolutionary distances. Based on indirect evidence gathered in *E. coli*, the *hypD* protein is assumed to take part in the incorporation of Ni into the active centre [Theodoratou et al., 2000].

hypE

It has been found in several bacteria. The protein encoded by this gene plays an important pleiotropic role in the formation of an active NiFe hydrogenase, but its specific function is not known. In *T. roseopersicina* it is located in the vicinity of *hypCD*.

hypF

This gene codes for a fairly large protein that has a central role in the assembly of NiFe hydrogenases. The homologous protein in *E. coli* was shown to have an acyl-phosphatase consensus sequence and a domain typical of enzymes performing O-carbomoylation [Paschos et al., 2001]. In addition, it contains a putative chaperone domain. These domains are clearly distinguishable in the translated hypothetical *T. roseopersicina* HypF protein, as well [Fodor et al., 2001]. Taken the various, conserved properties together, HypF is believed to be the protein that synthesises and incorporates the CO and CN ligands into the active centre. When *hypF* is deleted, none of the NiFe hydrogenases are synthesised in an active form. The Δ*hypF* mutant of *T. roseopersicina* produces large amounts of H_2 under nitrogen fixing conditions, indicating that the majority of the NiFe hydrogenase activity is in the H_2-uptake direction *in vivo*. The Δ*hypF* mutant of *T. roseopersicina* was the first direct evidence showing that this bacterium can be "engineered" to release significant amounts of biohydrogen, although in this case, the evolved H_2 originated predominantly from the nitrogenase complex [Fodor et al., 2001].

hupK

Interestingly, this gene has been found in a very limited number of bacteria so far [Cammack et al., 2001]. In those instances where *hupK* is present, it is indispensable for the formation of the active enzyme. Based on circumstantial evidence, a role in "handling" the Fe atom has been assigned to *hupK*, although this does not explain how other strains, lacking HupK, assemble the active centre of their NiFe hydrogenases. We have demonstrated unequivocally, that *hupK* takes part in the targeting of the enzymes into the membrane (G. Maróti et al., unpublished results), hence it provides the "finishing touches" on the membrane-bound hydrogenases, rather than inserting a metal atom at an earlier stage. This could only be demonstrated in *T. roseopersicina*, where the Δ*hupK* mutant did not produce the membrane-associated HynSL and HupSL, while the soluble enzymes were formed intact. Indeed, a closer look at the few other systems known from the literature revealed that bacteria, which do not contain membrane-bound hydrogenase, do not have *hupK*, and the ones synthesising membrane-bound enzymes do.

hydD/hynD

The *hydD/hynD* [Theodoratou et al., 2000] protein is most likely an endopeptidase. Its function is to clip off a peptide segment from the C-terminus of the stable hydrogenase large subunit *hynL*. When the heterobinuclear metal centre, together with the CN and CO diatomic ligands, is properly inserted into the protein core, the C-terminal processing cuts off a peptide necessary to keep the complex open. After elimination of the C-terminal peptide, the remaining tail of the *hynL* polypeptide flips over and locks the active centre into the protein matrix. The complex metal centre can only be removed from the assembled hydrogenase after an irreversible inactivation of the enzyme. It is worth noting that in the *T. roseopersicina hydD* gene, no typical transcriptional start codon or ribosome binding site could be identified yet, suggesting that a thorough functional study is warranted.

HETEROLOGOUS COMPLEMENTATION STUDIES

The genes identified, sequenced and partially characterised using transposon mutagenesis and sequence alignment need also to be analysed for their physiological function. One straightforward method for the functional tests is to transfer the gene in question, cloned on a suitable plasmid vector, into a homologous or heterologous host cell, which lacks this gene and check for the restored hydrogenase activity. The process is called complementation. Homologous complementation takes place when the gene originates from the same bacterial strain as the host cell (but of course the chromosomal copy of the gene is deleted in the host). Heterologous complementation occurs when the gene is introduced into a bacterium different from the strain the studied gene originates from. Since *T. roseopersicina* strains lacking the accessory genes are not available yet, most of the complementation experiments have been done using the appropriate *E. coli*, *R. capsulatus* and/or *R. eutropha* deletion strains, obtained from our collaborating partners within the European basic research network COST Action 841 (Prof. Barbel Friedrich, Humboldt University, Berlin, DE; Prof. Paulette M. Vignais CEA/CENG Grenoble, FR; Prof. August Böck, University of Münich, DE). It is reasonable to assume that the accessory genes, particularly the pleiotropic ones will be functionally active in the heterologous host cell. The experiments have been completed in the case of *hypC*$_1$, *hypD* and *hypF*. *HypD* from *T. roseopersicina* could not restore the corresponding function in *E. coli*, indicating significant differences between the "assembly - line" of the two bacteria.

In the case of *hypC1*, a strong background activity is observed in *E. coli*. *HypC* only participates in the assembly of the 3rd hydrogenase of *E. coli* [Drapal and Böck, 1998], the other two hydrogenases remained active in the Δ*hypC E. coli* strain and interfered with the measurements. This observation questions the truly pleiotropic nature of *hypC* in *E. coli* and, corresponding with these doubts, we have found a second *hypC* gene in *T. roseopersicina*. This suggests distinct *hypC* genes for the assembly of, at least some of, the Ni-Fe hydrogenases both in *T. roseopersicina* and in *E. coli*.

Complementation with *hypF* gene from *T. roseopersicina* in a Δ*hypF* strain of *R. capsulatus* was successful, a clear demonstration that a functionally active form of this *Thiocapsa* gene product is synthesised by the *R. capsulatus* cells from the „foreign" template. The same experiment using a Δ*hypF E. coli* strain resulted in barely detectable complementation. We conclude that there must be strain dependent variations in the complementation capacity and that the most thoroughly studied bacterium, *E. coli*, may not be the best choice for such complementation studies of hydrogenase assembly and biosynthesis.

CONSTRUCTION OF DELETION MUTANTS

In order to study the role(s) of the accessory genes, strains, in which these genes are knocked out from the genome, also have to be constructed in *T. roseopersicina*. Such mutants are generated by site directed deletion mutagenesis and the deletions have to be made „in frame" in order to make sure that genes, downstream from the mutated one, are not harmed and can function properly. This is rather tedious work and we only succeeded in the case of Δ*hupK* so far. Other deletion constructions have been also produced and the verification of the in-frame deletion is in progress. As mentioned above, studies using the Δ*hupK* strains of the wild type and various hydrogenase deficient *T. roseopersicina* strains revealed the membrane targeting function of the *hupK* accessory protein.

METHANOTROPHIC HYDROGENASES

Methane-oxidizing bacteria (methanotrophs) have attracted considerable interest over the past twenty years because of their potential in producing bulk chemicals (e.g. propylene oxide) and single-cell protein and for use in biotransformation [Dalton et al., 1995]. Methanotrophs are unique in that they only grow on methane, although some will also grow on methanol. Methanotrophs oxidize methane using the enzyme methane monooxygenase (MMO) [Stanley et al., 1983]. The soluble enzyme complex (sMMO) is present in some but not all methanotrophs [Murrell and Dalton 1992]. The sMMO is a remarkable enzyme in that it can also oxidize a large number of other substrates such as alkanes, alkenes and even aromatic compounds. The other form of MMO, found in all methanotrophs, is the membrane-bound or particulate form (pMMO) [Nguyen et al., 1998]. It has narrower substrate specificity than sMMO.

Both MMO enzymes require reducing power for catalysis. The *in vivo* electron donor for the sMMO is NADH [Lloyd et al., 1999]. Under physiological conditions, the reducing power is supplied by the further oxidation of the methanol (via formaldehyde and formate to CO_2) produced by the MMO. Since biodegradation processes using MMO are cooxidation processes, alternative ways of supplying reducing power are needed. A possible alternative is hydrogen.

Little is known about hydrogenases of methanotrophs. De Bont [1976] reported hydrogen-uptake activity in *Methylosinus trichosporium*, which was induced during N_2 fixation. The presence of an uptake hydrogenase was suggested since acetylene reduction by whole cells could be driven by hydrogen. Constitutive hydrogen-evolving activities (1 nmol·min^{-1} (mg dry wt·cell^{-1}) from formate under anoxic conditions were reported for *Methylomicrobium album* BG8 and *Methylosinus trichosporium* OB3b [Kawamura et al., 1983]. Chen and Yoch [1987] detected distinct constitutive and inducible hydrogen-uptake activities in *Methylosinus trichosporium* OB3b. Hydrogen-uptake activity in *Methylosinus trichosporium* OB3b was shown to be able to supply reducing power for both sMMO and pMMO activities [Shah et al. 1995]. Hydrogen driven propylene oxidation by *Methylococcus capsulatus* (Bath) was demonstrated by Stanley and Dalton [1992], but the mechanism was not investigated in detail. There exist at least two NiFe hydrogenases in *M. capsulatus* (Bath) [Hanczár et al., 2002]. The genes encoding a membrane-bound NiFe hydrogenase has been sequenced and characterized [Csáki et al., 2001].

Hydrogen production by whole cells

Washed cells of fermenter-grown *M. capsulatus* (Bath) produced hydrogen from sodium formate, and dithionite reduced redox dyes. This hydrogen could be evolved either by a hydrogenase or by nitrogenase activity. If the nitrogenase complex produced hydrogen, the effect should be observable under nitrogenase-derepressed conditions. The cells were, however, grown in NMS (nitrate mineral solution), and nitrate is known to repress nitrogenase synthesis [Murrell and Dalton 1983]. The same experiments were repeated in the presence of ammonium chloride at a concentration (4 mM) that was also known to inhibit nitrogenase activity [Murrell and Dalton 1983]. No decrease in hydrogen evolution was observed, which confirmed that hydrogen evolution from these substrates was not linked to the nitrogenase complex. Hydrogen production from formate was detected previously in two methanotrophs [Kawamura et al., 1983], but the enzymes involved had not been identified. Taking these observations together, a formate dehydrogenase-linked hydrogenase activity should be assumed in *M. capsulatus* (Bath).

Multiple hydrogenases

The cellular location of hydrogenase activities, measured either in the hydrogen evolution or in the hydrogen uptake direction, suggested the presence of more than one hydrogenase activity in *M. capsulatus* (Bath). Hydrogen-evolving activity was detected in both membrane and soluble fractions using reduced viologen dyes, but only the enzyme activity in the soluble fraction could accept electrons from NADH or dithionite-reduced methylene blue [Hanczár et al., 2002]. Similarly, both fractions showed hydrogen-uptake activity with the viologen mediators, but only the soluble fraction could reduce NAD^+. Notably, the hydrogenase activity assays were negative in both directions using $NADP^+$, and in general both fractions showed higher activity in the hydrogen uptake direction than in hydrogen evolution.

The remarkably distinct properties of the membrane and soluble fractions suggested that an NAD^+-reducing hydrogenase was present in the soluble fraction and that a distinct enzyme was also present in the membrane fraction which preferred methylene blue and did not react with NAD^+ or NADH.

Membrane-bound hydrogenase

The hydrogenase activity detectable in the membrane fraction was significantly lower under all assay conditions, which could be due to the uneven distribution of proteins in the crude fractions. The outstanding hydrogen-uptake activity in the presence of the methylene blue redox mediator should be noted. Benzyl viologen and methyl viologen also function as a redox mediator, but they are one or two orders of magnitude less efficient than methylene blue. The hydrogenase activities did not change significantly upon switching to nitrogen-fixing growth conditions, except for benzyl-viologen-mediated hydrogen-uptake activity, which increased five-fold in comparison to the nitrogenase repressed cells. Cells exposed to hydrogen during growth, in an attempt to induce expression of hydrogenase [Cammack et al., 2001], showed no significant increase in hydrogenase activity. The apparent K_m for hydrogen was 0.8 mM. In summary, the membrane bound uptake-hydrogenase had a relatively high affinity for hydrogen and was apparently expressed constitutively under the routine growth conditions used here.

As this was the first indication of the presence of a membrane-bound hydrogenase in a methanotroph, the biochemical observations were corroborated with molecular biological data The *hupSLECD* gene cluster has been cloned and sequenced from *M. capsulatus* (Bath). The cluster coded for a typical membrane-bound [NiFe] hydrogenase [Wu and Mandrand 1993] and deletion of the gene cluster resulted in a *ΔHupSL* phenotype. The *ΔHupSL* mutant [Csáki et al., 2001] retained practically all of the hydrogen-evolving activity in whole cells, but the lack of the hydrogen uptake activity with methylene blue in whole cells was demonstrated. Both hydrogen-uptake and hydrogen-evolution activity of the membrane fraction disappeared [Hanczár et al., 2002]. The residual hydrogenase activity is located in the soluble fraction in the mutant, which substantiates the presence of at least two hydrogenases in *M. capsulatus* (Bath).

Soluble hydrogenase

The existence of a distinct soluble hydrogenase was demonstrated with hydrogenase activity measurements. In order to determine whether this activity resided in the cytoplasmic or periplasmic compartment, the hydrogenase activity of the periplasmic and cytoplasmic fraction was measured separately; the activity was found in the cytoplasmic fraction (data not shown). The maximal hydrogen-uptake activity was measured using NAD^+ as an electron acceptor (25.5 nmol hydrogen oxidized·min^{-1}(mg protein)$^{-1}$, although both uptake and evolution hydrogenase activity could be demonstrated using methyl viologen, benzyl viologen and methylene blue. It should be noted that the low activity in the presence of methylene blue may be due to the leakage of some membrane-bound hydrogenase into the soluble fraction, because no corresponding hydrogenase activity could be measured in the Δ*HupSL* mutant. Formate-dependent hydrogen evolving activity was measured in whole cells of the wild type and of the Δ*HupSL* mutant. This observation indicated that the formate dehydrogenase and the soluble hydrogenase worked together during this *in vivo* measurement.

Hydrogen-driven MMO activities

As only the soluble hydrogenase utilized NADH, *in vivo* assays could be applied to investigate this activity further. Hydrogen-driven MMO activities were measured to obtain information on the *in vivo* function of this hydrogenase. The apparent K_S for hydrogen was again 0.8 mM in both assays. Maximal rates of MMO activities were 140 nmol·min^{-1}

(mg cell protein)$^{-1}$ for the sMMO and 260 nmol·min^{-1} (mg cell protein)$^{-1}$ for the pMMO. Positive control assays with 20 mM sodium formate as electron donor confirmed that maximum rates were not limited by NADH, but by the activity of the MMOs. Cells grown in the presence of 5% hydrogen or under nitrogen-fixing conditions (thus generating hydrogen *in situ* inside the cells) did not show any difference in V_{max} or K_S of hydrogen-driven MMO activities.

Raising the incubation temperature from 45 to 57°C did not bring about a pronounced increase of the hydrogen-driven pMMO activity. This preliminary observation indicated *in vivo* heat stability of the hydrogenase and pMMO activities. The temperature dependent difference in the solubility of hydrogen may also explain the small activity difference, particularly as similar results were obtained for the hydrogen-driven sMMO activity.

Most hydrogenases are sensitive to oxygen exposure [e.g., see Cammack et al., 2001]. The interaction between O_2 and the functionally active hydrogenase could be studied only by indirect methods, such as the hydrogen-driven MMO activity assays, since direct hydrogenase assays (both hydrogen evolution and hydrogen uptake ones) require the complete absence of oxygen. Increasing the oxygen concentration to 10 % (v/v) clearly had a positive effect. Further increase of the O_2 concentration to 15 % still did not cause any drop in either of these sMMO or pMMO activities [Hanczár et al., 2002]. Increased oxygen concentrations are likely to improve the rate of product formation through the methane monooxygenases rather than the hydrogenase(s). This suggests that the activity of the MMO itself is the rate-limiting factor in the combined (hydrogen-driven MMO) activity.

Utilisation of hydrogen metabolism in biotechnological applications

Hydrogen evolution by intact bacterial cells is frequently observed in nature. In microbial ecosystems the role of these microorganisms is creation and maintenance of anaerobic, reductive environment as well as supplying the universal reducing agent, molecular hydrogen. Gaseous hydrogen is usually not released from the natural ecosystems unless there is an excess of reductive power which needs to be disposed of in order to ensure the optimal metabolic and growth equilibrium in the population. H_2 generated *in vivo* by hydrogen forming bacteria is utilized by hydrogen consuming members of the microbiological community. Hydrogen is transferred to the recipient micro-organism very effectively by interspecies hydrogen transfer. The molecular details of this process are not fully understood, but its significance in safeguarding the optimum performance of the entire ecosystem and the delicate regulatory mechanisms should be appreciated. In the mixed population bacterial systems presented here the advantages of interspecies hydrogen transfer are exploited [Kovács and Polyák, 1991].

Biogas

Decomposition of wastes anaerobically to form biogas is one of the earliest applications of biotechnology. Let us turn first briefly to the microbiology of the biogas formation. It is well known that three distinct microbe populations take part in the anaerobic digestion process. These microbe populations are the polymer degrading, so-called hydrolyzing bacteria, the acetogens and the methanogens. The first group, the polymer degraders attack the macromolecules using extracellular enzymes and producing intermediers. Because of its abundance in Nature, cellulose is the main substrate for hydrolyzing bacteria. The acetogens then use these sugars and oligosaccharides and produce organic acids, like acetate, succinate, formate, propionate, and carbondioxide. The third group is the methanogens.

These microorganisms belong to the Archaebacteria and thus possess unique molecular

and cellular properties. They produce methane using acetate, hydrogen and carbondioxide.

Our recent research results show that H_2 has an important role in the anaerobic fermentation. Although a product of an intermediate step, only traces of hydrogen is found in the final product, biogas. This suggests that hydrogen may be a rate limiting substrate for methanogens. In order to check this hypothesis, the biogas forming natural consortium of microorganisms has been inoculated with a suitably selected hydrogen producing strain. Theoretically, there are three possibilities for the outcome of such experiment as follows.

1. The presence of the hydrogen producing bacterium has no effect on biogas formation. This would indicate that the hypothesis was wrong and biogas formation by methanogens is not limited by the amount of hydrogen available.
2. Hydrogen accumulates in the head space of the anaerobic fermentor.
   This would suggest that although the hypothesis was wrong and biogas formation is not limited by hydrogen, the caloric value of the biogas formed could be increased via the hydrogen component of the enriched biogas.
3. There is no hydrogen appearing in the final product but the amount of biogas formed increases. This would be interpreted as a proof for hydrogen being the rate-limiting step in biogas formation, indeed.

During the anaerobic biodegradation hydrogen concentration is reduced to a much lower level than that of acetate. In addition, the hydrogen partial pressure can change rapidly within a few minutes. We have shown that under these circumstances addition of hydrogen producers to the system brings about advantageous effects for the entire microbiological methanogenic cascade. The decomposition rate of the organic substrate, which was animal manure in our first experiments, increases and both the acetogenic and methanogenic activities are amplified. In laboratory experiments some 2.6-fold intensification of biogas productivity has been measured. The manure was inoculated with a vigorously hydrogen producing bacterium strain two times, first at the beginning of the fermentation, and seven months later. Methane production increased after the inoculation and the accumulated biogas production increased significantly.

In is to be noted that similar mechanisms have been suggested earlier and tests were carried out using hydrogen added externally from a gas cylinder. All those experiments failed and showed a strong inhibitory effect of hydrogen on methanogenesis. Indeed, too much hydrogen inhibits the metabolism of methanogens. Supplying the reducing power using the help of a bacterium, however, balances the microbial system and brings about the beneficial effect of additional hydrogen. Interspecies hydrogen transfer between the hydrogen producing and consuming microbial partners plays a determining role in the effectiveness of the biogas intensification process.

Some other experiments at various scales and using distinct organic waste sources have been carried out. Volumes between $0.1 m^3$ and 10,000 m^3 were used. In every experiment the methane production increased. Best results were obtained with pig manure. In this case the biogas production increased to 200 %. The experiments thus proved that it was possible to increase the gas production using intensified microbiological biomass decomposition.

The pig slurry contains only about 5 % dry weight, which can be elevated by adding biomass from energy plants. The energy plants' biomass content is about 35 %, so it is possible to increase the solid content of the biomass input using energy plants. The most

suitable energy plants in moderate continental climate areas, such as Hungary, are sweet sorghum and Jerusalem artichoke, which accumulate sugar as storage material. Taking into account that pig manure is about 5 % dry weight, while plant biomass is about 35 %; the results of basic calculations are given as follows.

Produced biogas (Nm^3/yr)	14,400
Caloric value (MJ/yr)	316,800
Exhaust (Nm^3/yr)	105,552
Manure requirement (m^3/yr)	302
Plant biomass requirement (tons/yr)	113
Effluent volume (tons/yr)	399
Effluent concentration (%)	11

We have studied the microbiological and biochemical aspects of the process for several years and identified weak points where significant improvement of the biogas production efficacy can be achieved. A major rate limiting step of the overall biogas formation reaction chain is interspecies hydrogen transfer and the availability of reducing power for methanogens in the complex microbiological populations. We have shown that an alteration of the bacterial population balance to facilitate interspecies H_2 transfer brings about pronounced beneficial effects: an increase of the biodegradation rate and biogas productivity. When compared to the currently employed technologies, the practical advantages include lower operational costs (smaller digesters and/or shorter retention times; more biogas) and decreased environmental stress upon discharging the digested material.

The technology can be easily appended to the existing systems. Optimization work as well as scale up included treatment of a household waste landfill sites of the city of Szeged, Hungary. The waste depository accumulates 300,000 m^3 of solid household waste and produces over 700,000 m^3 of biogas annually. An average biogas productivity over 30% has been registered. In an other field demonstration, a waste water sludge digester of 2500 m^3 volume has been treated. In this case a biogas productivity of 80% has been recorded. Additional scale up experiments were done in an EUREKA project (EU1241, (Fig. 5)) where the beneficial effect of interspecies hydrogen transfer is used in anaerobic treatment of animal waste.

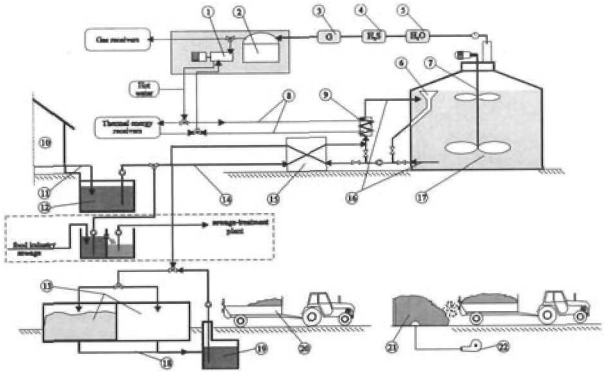

Figure 5. Scheme of the biogas production plant.
1. 380 V generator, 2. biogas tank, 3. gasmeter, 4. desulphurization, 5. dehydrator, 6. overflow, 7. mixer, 8. water pipeline, 9. heat exchanger: water/manure, 10. piggery, 11. manure transporting pipeline, 12. preliminary tank, 13. manure chambers, 14. feeding pipelines, 15. heat exchanger: manure/manure, 16. mixing pipeline, 17. fermentation chamber, 18. drainage, 19. reflux tank, 20. dung spreader + tractor, 21. composting plate, 22. fan.

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APPLICATION OF HYDROGENASE FOR RENEWABLE ENERGY MODEL SYSTEMS

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INTRODUCTION

Hydorogen is considered as one of the most attractive and promising fuel and intermediate energy carrier for future energetics. Renewable energy economy could be based on the balance between production and consumption of hydrogen. In this paper some hydrogenase containing model systems for transformation and accumulation of energy are outlined.

The main source of energy for life on the Earth is solar energy. The solar radiation reaching the earth surface exceeds any predictable energy requirements of mankind. However, the efficiency of conversion and accumulation of this energy by photosynthetic microorganisms is rather low. Solar energy could be used directly to split water into hydrogen and oxygen by biological and photochemical systems. According to thermodynamics the light quanta with $\lambda < 1000$ nm (>1.23 eV) are required for this reaction. It means that all photosynthetically active solar radiation is acceptable for hydrogen photoproduction by photosynthetic organisms and artificial model systems.

At present, several stable photocatalytic systems for production of hydrogen from water and organic compounds are made of semiconducting oxides and suitable proton reducing catalyzer. An efficient electron transfer between inorganic semiconductor and bacterial hydrogenase was shown to result in hydrogen photoproduction.

The oxidation of hydrogen in fuel cells provides clean energy and water as the only by-product. Application of hydrogenase for hydrogen electrode is able to improve the characteristics of the fuel cells. Thermostable hydrogenase from *Thiocapsa roseopersicina* is an appropriate catalyst for development of several systems for production and transformation of renewable energy based on molecular hydrogen.

PHOTOPRODUCTION OF HYDROGEN BY INORGANIC SEMICONDUCTOR TiO_2 – HYDROGENASE MODEL SYSTEM

The ability of H_2 photoproduction as a result of photoinduced electron transfer from TiO_2 particles to hydrogenase was demonstrated in 1976 by Krasnovsky at al. [1]. H_2 was produced under irradiation of TiO_2 suspension containing hydrogenase from *T. roseopersicina*,

Tris buffer as an electron donor and methyl viologen (MV) as an electron mediator (Fig. 1). Without MV the reaction rate was by two orders lower than that in the presence of electron mediator. That was due to low efficiency of the direct transfer of the photogenerated electrons from semiconductor to the enzyme molecules. Further study non-mediator H_2 photoproduction showed that addition of $CaCl_2$ enhanced significantly the rate of this process. At the optimal $CaCl_2$ concentration (1.6 mM) the rate of H_2 photoproduction was comparable with that observed in the presence of MV [2]. $CaCl_2$ also activated H_2 production in the presence of methyl viologen. However, in this case, $CaCl_2$ caused only a 3-fold increase in the reaction rate, while without an electron mediator the increase was greater than 30-fold.

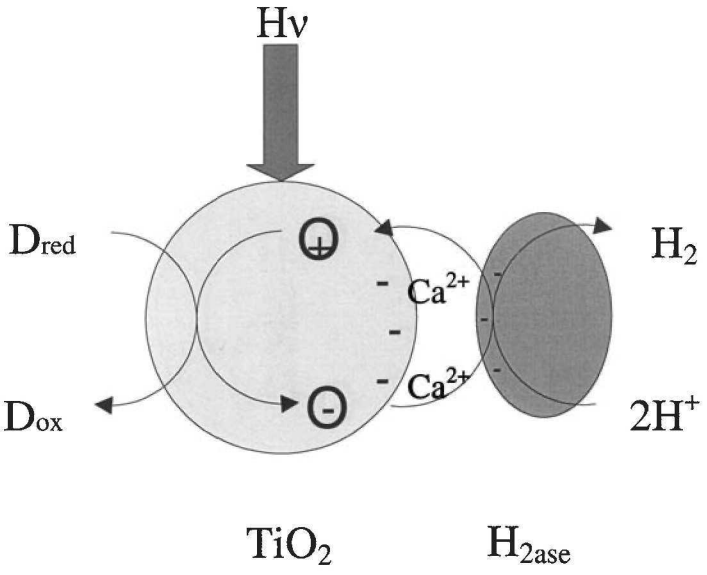

Figure 1. H_2 photoproduction by TiO_2 - hydrogenase model system.

The activation of H_2 photoproduction by $CaCl_2$ was observed not only with Tris buffer as an electron donor but with sucrose, dithiothreitol, or methanol as well. $BaCl_2$, like $CaCl_2$, enhanced the rate of H_2 photoproduction by a factor of about 30. Chlorides of monovalent metals increased the reaction rate in the system Tris-TiO_2-hydrogenase insignificantly [2].

The TiO_2 suspension was able to absorb proteins including hydrogenase. Depending on pH of the solution, the hydrated surface of TiO_2 is charged either positively or negatively due to amphoteric nature of surface OH groups. In the presence of Tris buffer (pH7.2) TiO_2 samples had negative charge, as well as hydrogenase molecules that prevented the absorption [2]. The bivalent cations adsorbed on TiO_2 particles may significantly decrease or change their negative charge and create conditions for binding of hydrogenase (Fig. 1). Hydrogenase adsorption induced by $CaCl_2$ was reversible in accordance with the electrostatic nature of enzyme-semiconductor interaction. Under optimal conditions for adsorption on TiO_2 particles the quantum yield of H_2 photoproduction in this system could reach 30 % with ultra violet light (λ = 365 nm). However the stability of the TiO_2 - hydrogenase system was low in

these conditions, probably, due to photodestruction of enzyme molecules.

PHOTOPRODUCTION OF HYDROGEN BY INORGANIC SEMICONDUCTOR CdS – HYDROGENASE MODEL SYSTEM

Hydrogen production was demonstrated in the system containing inorganic semiconductor CdS, the electron donor and hydrogenase under visible light. The character of interaction between hydrogenase and semiconductor in this system has a complicated nature. Divalent cations, which significantly increased the absorption of hydrogenase on TiO_2, had no or very small effect on binding the enzyme on CdS. The process of the sorption/desorption of hydrogenase on CdS surface was controlled by the types and pH of the buffer used.

Under illumination of CdS suspension in the absence of hydrogenase, metallic Cd was formed on the semiconductor surface. In these experiments dispersed CdS powder from "Aldrich" or CdS produced by the mixing of $CaCl_2$ and Na_2S solution was employed. Up to 20% of Cd^{2+} of CdS matrix could be reduced to metallic cadmium in the presence of formate as an electron donor. The photogenerated Cd^0 was oxidized as an electron donor for H_2 production after addition of hydrogenase (Fig. 2). Optimal temperature for H_2 photoproduction by this system was 75°C and pH was about 5.0 that coincided with optimal conditions for catalytic action of *T. roseopersicina* hydrogenase. Cadmium metal photoproduced on the surface of semiconductor participates in H_2 photoproduction in two ways: as a transfer of the conduction band electrons to the enzyme active site and as a substrate in the enzymatic hydrogen production. Hydrogenase added to preliminary illuminated CdS suspension in the presence of electron donors could oxidize photoreduced metallic Cd in the dark [3]. Thus, semiconductor – hydrogenase systems may produce hydrogen through direct photoinduced electron transfer from the semiconductor to the enzyme or indirect transfer mediated by the metal, photoreduced on the surface of CdS particles.

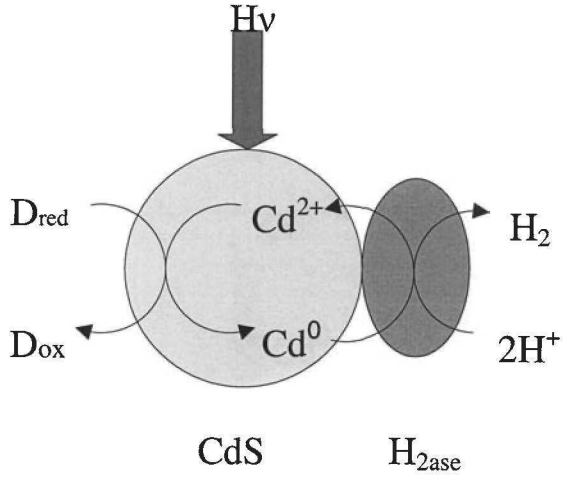

Figure 2. H_2 photoproduction by CdS - hydrogenase model system.

Hydrogen production process by direct photolysis of hydrogen-containing compounds may be based on different types of photosensitizers in parallel with inorganic semiconductors. The model systems for photoinduced hydrogen production with porphyrins and hydrogenase were developed [4].

PRODUCTION AND OXIDATION OF HYDROGEN BY HYDROGENASE WITH METAL AS ELECTRON DONOR/ACCEPTOR

The role of hydrogenase in anaerobic biocorrosion of metals is the subject of wide discussion. Bryant and Laishley [5] demonstrated that hydrogenase may be involved in anaerobic biocorrosion of mild steel. *T. roseopersicina* hydrogenase accelerated anaerobic oxidation of several metals which have redox potential of the Me/Me^{2+} couple near or more negative than the potential of hydrogen electrode. The oxidation of metals is accompanied by hydrogen production (Fig. 3). The most intensive hydrogen production was observed in the presence of metallic iron or zinc in the system containing hydrogenase and MV as electron mediator between metal and enzyme. Hydrogenase stimulated H_2 evolution 3-10 times depending on dispersity, surface state of metal powder, the temperature and other parameters. To study H_2 evolution over a wide pH range (2.5-10), the metallic Zn was proposed as alternative reductant of MV instead of dithionite [6]. Metallic Cd also supported H_2 production in the system: metal - methyl viologen - hydrogenase. As it was already mentioned direct electron transfer between metal and hydrogenase was demonstrated in the system containing metallic cadmium. The hydrogen production from suspension of powder of metallic Cd was measured with hydrogen electrode. In control without enzyme H_2 production from Cd^0 was very low. Upon addition of hydrogenase to suspension of cadmium powder the rate of H_2 production was increased by 3-5 times with further increasing after addition of MV. The non-mediator H_2 production from Cd^0 reached 35 % relative to that in the presence of MV in the pH region from 4.0 to 7.0.

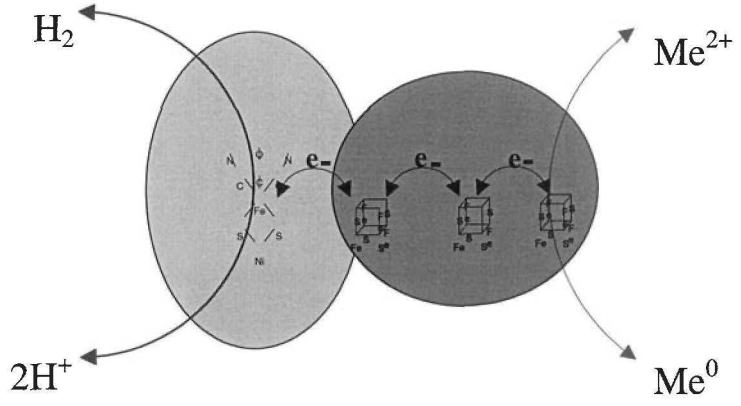

Figure 3. Proposed interaction of hydrogenase with metal.

The hydrogen production from metallic nickel in such system occurred with low rate at pH below 5.0 and stopped after several minutes. At pH above 7.0 hydrogenase catalyzed the

reverse reaction of Ni^{2+} reduction to metallic nickel in H_2 atmosphere that corresponded with redox potential of Ni/Ni^{2+} couple. From thermodynamic point of view hydrogenase can reduce several metal ions with positive redox potential in the H_2 atmosphere. However the reduction of Ni^{2+}, Ru^{2+} and Pd^{2+} was only observed. Other metal ions can not be reduced by hydrogenase. One of the possible reasons for that is the strong inhibition of hydrogenase by metal ions. We have studied the effect of some ions on the activity of *T. roseopersicina* hydrogenase. As it has been shown there are two types of inhibition by metal ions [7]. Most of metal ions inhibited the hydrogenase in the reaction of H_2 oxidation reversibly and competitively with respect to MV. The affinity of these metal ions to the enzyme increased significantly with the increase of pH when the charge of hydrogenase became more negative. These data indicate that reversible inhibition of hydrogenase by cations of metals proves electrostatic character of their interaction. The ions of some heavy metals (Cu^{2+}, Hg^{2+}, Ag^{+}) inhibited the hydrogenase activity irreversibly and uncompetitively with respect to methyl viologen. In the presence of these metal ions the absorption of hydrogenase at 400 nm was decreased that indicated the destruction of the FeS cluster in the enzyme [7].

The effect of several polypeptides with different charges on hydrogenase activity was investigated to clarify the role of surface charge on substrate and inhibitor binding. It has been found that polypeptides with positive charges have strong inhibitory effect. For example, the poly-lysine 20 is a strong competitive inhibitor versus MV and has k_i about 2μM. The inhibitions decreased with decreasing positive charge of polypeptide. The mixed poly-lysine-leicine had lower positive charge and in this case k_i was about 8μM. The uncharged polypeptides or polypeptides with negative charge did not show any inhibition of *T. roseopersicina* hydrogenase. These data demonstrate that electrostatic interactions play an important role in binding both the substrates and the inhibitors with enzyme.

HYDROGEN ENZYME ELECTRODE FOR RENEWABLE ENERGY

The hydrogen enzyme electrode is a device, which able to activate reversibly hydrogen molecule using catalytic activity of hydrogenase for energy interconversion between H_2 and electricity. The effective electron exchange between the enzyme active site and electrode surface is required for function of this device, for example, in water electrolyzer, hydrogen fuel cells and other energy conversion systems. Several approaches were used to develop hydrogen enzyme electrodes based on direct and mediated bioelectrocatalysis by hydrogenase from *T. roseopersicina*. The bioelectrocatalysis of hydrogen oxidation and evolution by hydrogenase absorbed on carbon black electrode was shown in the absence of any diffusion-free or immobilized mediator [8].

For further improvement of hydrogen enzyme electrode the commercial carbon filament materials were used as an electrode matrix. Such type of materials are accessible and well characterized, that provides the reproducibility of the results. A procedure for hydrogen enzyme electrode preparation included the pretreatment of electrode support with sulfuric acid followed by enzyme immobilization. This procedure is a critical step, since initially carbon filament material is completely hydrophobic [9].

After immersion of the enzyme electrode in neutral buffer solution saturated with H_2, the equilibrium hydrogen potential was achieved. The enzyme electrode based on carbon filament material with immobilized *T. roseopersicina* hydrogenase was characterized by high current of hydrogen oxidation. The hydrogen enzyme electrode as compared to platinum electrode of the same geometric area was shown to possess similar electrocatalytic activity. However, when recalculated per molecule of the catalyst, the efficiency of the enzyme was two orders of magnitude higher [9].

A critical factor for biotechnology application is the stability of the enzyme electrode. Hydrogenase immobilized into carbon filament material has high level of both operational and storage stability. Even after the half year of storage with periodical testing, the enzyme electrode preserved more than 50 % of its initial activity [9,10]. Thus, it is possible to achieve appropriate stability of the enzyme electrode, suitable for hydrogen fuel cells development.

The most important problem of platinum-based hydrogen electrodes in fuel cells is their poisoning by carbon monoxide. At the present hydrogen gas produced industrially by conversion of fossil fuels contains high level (> 1.0 %) of CO, which poisons platinum surface. However, even in the presence of 0.1 % carbon monoxide in hydrogen the platinum electrode lost 99 % of its activity after 10 min of operation [11]. On the other hand, *T. roseopersicina* hydrogenase is rather stable to the effect of CO. The inhibition of this enzyme was significant only at CO content above than 1.0 % and was completely reversible even after exposing to 100 % CO [12].

Hydrogenase based enzyme electrode was not inhibited, when CO content in the mixture was less than 0.1 %. In the presence of 1 % CO the rate of hydrogen oxidation was decreased by 10 % and zero-current potential was shifted positively for 30 mV. The steady-state currents were achieved in a few minutes [10]. An important advantage of the hydrogen enzyme electrode is completely reversible nature of inhibition by CO. Like the soluble hydrogenase the enzyme electrode recovered 100 % of its initial activity as soon as the atmosphere of pure carbon monoxide was changed back to hydrogen.

Different electrode supports and methods of hydrogenase immobilization may be applied for the development of new energy conversion systems between electricity and hydrogen. Recent progress of molecular handling of hydrogenase including the Langmuir-Blodgett technology could provide a way to design the effective electrodes for hydrogen oxidation or production [13].

HYDROGENASE IN ENERGY SAVING AND ENVIRONMENTAL PROTECTING SYSTEMS

Table 1 presents biotechnological potential of hydrogenase employing different activities of this enzyme. Besides direct application in the energy conversion systems hydrogenase is a useful tool to reduce the energy consumption and protect the environment. Actually, the ability of hydrogenase to reduce reversibly some compounds like metal ions, viologens and other electron acceptors could be applied for development of safe and convenient hydrogen energy accumulator. If this compound is well soluble in water, its concentrated solution would be an efficient H_2 storage medium. Such an approach was experimentally proven using methyl viologen as a hydrogen binder and immobilized hydrogenase as a catalyst. An 0.5 M aqueous solution of MV accumulates 240 times as much H_2 as pure water dissolves under the same pressure. The hydrogenase provides the charge-discharge cycle within a reasonable time [14].

Table 1. Biotechnological potential of hydrogenase

Hydrogen production
Photocatalytic systems for H_2 production.
Hydrogen enzyme electrode for H_2 electrosynthesis.
H_2 production from some metals and reduced compounds.
Hydrogen consumption
Hydrogen enzyme electrode for fuel cells.
Amperometric biosensor for hydrogen.
Accumulator for hydrogen energy.
Biosynthesis of fine chemicals.
Treatment of waste water polluted by heavy metals.
Hydrogen-deuterium exchange
Preparative separation of hydrogen isotopes.
Detritiation of cooling water in nuclear power plants.
Biosynthesis of chemicals labelled with D or T.

Different approaches for enzymatic synthesis of fine chemicals using H_2 as the ultimate reducing agent were suggested [14]. Hydrogenase and some specific enzymes, coupling via cofactors, carry out the synthesis under soft conditions. Such multi-enzyme systems for production of fine chemicals have high specificity and should consume less energy as compared with usual chemical synthesis.

High hydrogen isotopes effect of hydrogen production by *T. roseopersicina* was observed when the activities were determined in usual and heavy water. The rate of H_2 production in H_2O was approximately 5 times higher than the rate of D_2 production in D_2O [6]. This effect could be applied for preparative production of heavy water and separation of hydrogen isotopes. During the long-time electrosynthesis of H_2 from H_2O on the hydrogenase electrode the heavy water will be accumulated in aqueous phase.

Tritium separation from hydrogen is required for detritiation of aqueous waters from nuclear power plants to avoid the environmental pollution by this radioactive isotope. Nobel metal catalysts have been traditionally employed for design of heavy water production and tritium separation systems [14]. High activity and oxygen stability of hydrogenase from phototrophic bacteria [6,15] in hydrogen-water isotope exchange reaction gives good perspectives for application of this enzyme as a catalyst in the production of heavy water and separation of hydrogen isotopes.

The metal-reducing ability of hydrogenase may help to remediate environmental pollution by some toxic metals. Hydrogenase has a potential for biological recovery of rare and expensive metals as well. Some hydrogenase containing microorganisms and their consortia with plants could be used for metal ions biosorbtion and redox transformation [16]. Enzymatic recovery of elemental palladium by using hydrogenase activity of sulfate-reducing bacteria was demonstrated [17].

CONCLUSION

The hydrogenase may be electrically wired with different semiconductors, metals and conducting materials as an electrode. This property of the enzyme is successfully used in design of different biomolecular device for renewable energy production and conversion systems based on molecular hydrogen as intermediate energy carrier. In many cases

hydrogenase as H_2-activating catalyst can replace the platinum and other noble metals.

At present the hydrogenase is very expensive and commercially unavailable enzyme for large scale application. There are three approaches to design rather cheap hydrogen-activating catalyst:

(1) The development of a new methods for hydrogenase production from industrially grown bacteria. The use of genetic modification of bacteria including overexpression of hydrogenase could significantly improve the yield of this enzyme.
(2) The application of whole cells of some microorganisms with high level of hydrogenase activity.
(3) The design of complex NiFe compounds which mimic active site of hydrogenase and have the catalytic activity comparable with pure enzyme.

The future research in the field of hydrogenase engineering would give a considerable progress in development of both renewable energy systems and molecular microsystems for medicine, electronics, analytical chemistry etc.

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II. Photosynthesis and Photobioreactor

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PHOTO-BIOLOGICAL HYDROGEN PRODUCTION BY THE UPTAKEHYDROGENASE AND PHB SYNTHASE DEFICIENT MUTANT OF *RHODOBACTER SPHAEROIDES*

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ABSTRACT

Rhodobacter sphaeroides KD131and its mutamt strainlacking uptake hydrogenase(Hup^-) and PHB synthase(PHB^-) have been studied on hydrogen production and cell growth under different culture conditions. Both strains produced hydrogen beginning from the middle of the logarithmic growth phase and continued until the cell concentration leveled out. The rates of hydrogen production were 1.62 ml H_2/ml-broth for the wild-type strain and 2.2 ml H_2/ml-broth for the mutant strain in 48 hr under the irradiance of 8 klux/m^2 using halogen lamps in the broth containing 30 mM D, L-malate and 8mM L-glutamate as carbon and nitrogen sources, respectively.

Malate and lactate were better carbon sources than starch, sucrose and glycerol for both strains for the hydrogen production and the cell growth. When the hydrogen production was limited in the culture broth containing D, L-malate as a carbon source, β-D-hydroxybutyrate accumulated for the first 20-25 hr of incubation by both strains and then was hydrolyzed with time. Approximately 55-60% of acetic acid was hydrolyzed in 48 hr by the wild-type strain and pH increased to 9.4. The increase in pH of culture resulted in lowering the hydrogen production. However, the mutant strain did not grow well under the same experimental condition.

pH ranges of 6.8-7.2 were the optimum for the cell growth and pH 7.4-7.6 for the hydrogen production. Irradiance of 7-8 klux/m^2 for the wild-type strain and 15-16klux/m^2 for the mutant were the optimum for both cell growth and hydrogen production. When the light intensity was increased higher than 50 klux/m^2, the cell growth was slightly inhibited and the cultures turned gradually to pink-red color for the first 48 hr, and eventually lost its color to white over 120 hr of incubation.

INTRODUCTION

Hydrogen is a clean and highly efficient fuel that results in water upon burning without generating CO_2. Among various hydrogen production technologies, biological conversion to hydrogen has received more attention as a sustainable technology since organic wastes, water and various gases are cheap starting materials. Photo-biological hydrogen production by purple non-sulfur bacteria is one of the most promising methods under photo-heterotropic conditions. The highest rate of photo-biological hydrogen production has been achieved with strains belonging to the purple non-sulfur bacteria *Rhodospirillaceae* and it also accumulates useful by-products in addition to hydrogen.

Over the years, many researchers have examined various aspects on different strains and reactors to improve the efficiency of biological hydrogen production. First, isolation and screening of strains for the high rates of hydrogen production resulted in many fresh or marine water photosynthetic bacteria, *Rhodopseudomonas palustris*, *Rp.* capsulate[1], *Rp.* strain TN3, *Rhodobacter rubrum*, *Rb. sphaeroides* OU001[2], RV[3], *Rhodobium marinum*[4], *Rhodobium sulfidophilum*. Second, membrane - bound uptake hydrogenase[5-7] and light-harvesting core antenna deficient or altered mutants[8-10] were mainly studied on *Rhodobacter sphaeroides* RV strain to improve the hydrogen production using recombinant DNA technologies. Numerous studies on accumulation of poly hydroxybutyrate (PHB) and hydrogen production in *Rhodobacter* strains also have been performed[11-13], even though the studies on PHB mutant for the hydrogen production were limited[14]. Third, various efficient closed photo-bioreactors were proposed for the hydrogen production such as biocoil, flat-plate and bag type reactors were examined in laboratory or outdoor experiments. In addition, a volume of 200 L tubular integral gas exchange reactor has been tested with *Spirullina* in Florence and Hawaii[15]. A floating type photo-bioreactor[16] for use on sea or lake was operated with *Rp. palustris* taking advantages of temperature control and mixing effects on sea- water in Tokyo Bay. Despite of many efforts on photo-biological hydrogen production over the years, we still need diverse collection of strains from nature and their improvements on efficient production of hydrogen for a practical use.

In this study, we examined a newly isolated *Rb. spaeroides* KD131 strain and a mutant derived from *Rb. spaeroides* KD131 by internal deletion of *Hup SL* and *Phb C* genes on their properties of growth and hydrogen evolution using different substrates and under different culture conditions.

MATERIALS AND METHODS

Microorganisms

Rb. sphaeroides KD 131 wild type and its mutant strains[17] were obtained from Dr. J. Lee (Sogang University, Korea).

Cultivation

Rb. sphaeriodes KD131 was pre-cultured in the modified Sistrom's broth containing $(NH_4)_2SO_4$ and L-aspartic acid as nitrogen sources and 35 mM succinic acid as a carbon source at 30°C for 20-24 hr under 7-8 klux/m^2 irradiance using halogen lamps(12V, 20W) by illuminating at one side of serum bottles. The pre-cultures were centrifuged to collect the cells and the cells collected were used as an inoculum for hydrogen production after adjusting the cell density to absorbance 0.5 at 660 nm in the Sistrom's broth containing 8.2 mM

L-glutamic acid and 30 mM malic acid under the same experimental condition as for the pre-culture. The 150 ml-capacity of a serum bottle was used for the pre-cultures and for the hydrogen production. Two types of 2.5 L-capacity flat-rectangular and vertical photo-bioreactors, which were made of glass for both flat sides and stainless steel for the edges or made of clear plastic acryl material, were used for the production of hydrogen and β-D-hydroxybutyrate.

Glucose, glycerol, sucrose, starch, acetate, malate and lactate were examined for the effect of carbon sources on the hydrogen production and the effects of light intensity were also examined under the different irradiances, such as 0, 3, 7, 15, 30, 50 and 110 klux/m^2, by adjusting the distance between the light source and the samples of *Rb. sphaeriodes* KD 131 cultures.

0.25 g of cuprammonium rayon fiber (2cm×180μl, Length×Diameter) was added to the 20 ml culture broth in 50 ml serum bottle to observe the effect of fibers for the H_2 production during photo-fermentation. The effect of fiber addition was also examined at the 2.5 L flat-rectangular photo-bioreactor.

Analysis

Hydrogen gas in the head-space of a reactor was analyzed by a gas chromatography (GC 14-B, Shimadzu Corp.) equipped with a molecular sieve 5A column using a thermal conductivity detector. The concentration of organic acids was measured using HPLC (SCL-10A VP, Shimadzu Corp.) fitted with an Aminex HPX-87H organic acid analysis column (Biorad Corp.). Reducing sugar contents were measured by the dinitrosalicylic acid (DNS) method, and starch contents were determined by the DNS methods after acid hydrolysis using 5.5 N HCl.

RESULTS & DISCUSSION

Growth and hydrogen production

A photosynthetic and hydrogen producing bacteria was isolated around the Kwang-Yang bay area in Korea where the fresh and sea water meet. The strain *Rb. sphaeriodes* KD 131 was identified comparing the DNA sequences that its nucleotide sequence of the 1,27-Kb DNA encoding part of 16S rRNA of the isolate showed 99.5% identity with those of *Rhodopseudomonas sphaeroides* 2.4.1 and *Rhodobacter sphaeroides* IFO12203. The mutant strain *Hup*$^-$ and *Phb*$^-$, derived from *Rb. sphaeroides* KD 131 have internal deletion of *Hup SL* and *Phb C* genes[17].

Hup$^-$/*Phb*$^-$ strain (mutant) grew faster than the wild type strain to reach the maximum cell concentration in 26 hr of incubation in the modified Sistrom's broth at 30°C under the 8-9 klux irradiance. Logarithmic growth phase of the wild type strain continued until 40 hr and the cell concentration at the maximum growth of the wild type strain was 1.2 times higher than the mutant (Fig. 1). Both strains produced the hydrogen beginning from the middle of the logarithmic growth phase and continued until the cell concentration has leveled out. It seems that polymers accumulated in the culture broth still provide the electron doners to evolve the hydrogen even though the carbon and nitrogen sources were exhausted. The rate of hydrogen production was 1.62 ml H_2 /ml-broth for the wild- type strain and 2.1 ml H_2 /ml-broth for the mutant at 48 hr of incubation under this experimental condition. Maximum hydrogen production of *Rb. sphaeriodes* KD 131 exhibited in the decreasing order of *Hup*$^-$/*Phb*$^-$, *Hup*$^-$, *Phb*$^-$ mutants and the wild type strain. When the culture conditions were optimized for the hydrogen production, *Phb*$^-$, *Hup*$^-$, and *Hup*$^-$/*Phb*$^-$ mutants produced

hydrogen 1.27, 2.06, and 2.39 times higher than the wild type strain, respectively. It indicates that the effect of double mutations in *Hup*$^-$ *Phb*$^-$ strain on the hydrogen production was additive by each mutation in *Hup SL* and *Phb C* genes, but the *Hup*$^-$ mutant contribute the more positive effect on the hydrogen production to the *Hup*$^-$/*Phb*$^-$ mutant than the *Phb*$^-$ mutant.

The initial pH 7.0 increased to 8.6-8.8 and then decreased to 7.4-7.7 for both strains, when succinic acid and $(NH_4)_2SO_4$ and L-aspartic acid were used as the carbon and nitrogen sources, respectively. The pH changes during photosynthetic incubation of these strain exhibited the similar trend when malic acid and glutamic acid were used as the carbon and nitrogen sources, respectively, in which pH slightly increased to 7.5-7.6 for 60-70 hr of incubation and then decreased to the 7.2-7.3 when hydrogen production stopped. Egoglu *et al.* [18] reported different observation in pH changes in the culture medium that a slight decrease in pH occurred during the cell growth and pH increased during the hydrogen production by *Rb. sphaeroides* OU 001 when the culture medium contained 30 mM L-malate and 2 mM sodium glutamate with the initial pH 7.5.

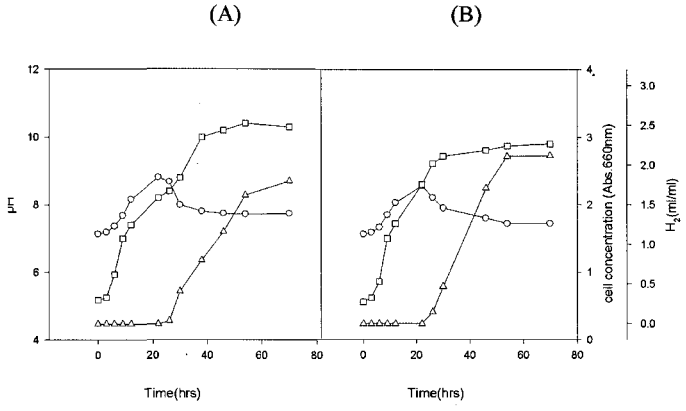

Figure 1. Growth curves of *Rb. sphaeroides* KD131 wild type (A) and mutant strain (B) in the modified Sistrom's broth at 30°C under the 8-9 klux irradiance.

△, total hydrogen produced; □, cell concentration; ○, pH

Carbon sources

Malate showed better cell growth and hydrogen production compared to the other carbon sources examined in this experiment. The mutant produced 2-2.2 ml H_2/ml-broth, which was equal to the approximately 55% conversion of malic acid when the theoretical conversion yield of hydrogen from 1M malate assumed 6 M hydrogen. The amount of hydrogen produced by the mutant strain was approximately 1.4 times more than the wild type strain using malate as a carbon source, whereas cell growth of the the wild type strain was better than the mutant showing the maximum cell concentration of the wild type and mutant strains, 3.10-3.30 and 2.95, respectively. When the isomeric mixture of malate was used as a carbon source, β-D-hydroxybutyrate (β-D-HB) was accumulated in the culture broth for the first 18-25 hr of incubation by both strains. Especially, when the culture conditions are not favorable for the production of hydrogen (Fig. 3), β-D-HB accumulated in high concentration and then hydrolyzed with time. The extent of β-D-HB accumulation was varied on the light intensities.

Table 1. Effect of carbon sources on H_2 production and cell growth of *Rb. sphaeroides* KD131 wild-type and mutant strains

	Wild-type strain				Mutant			
	pH	Cell conc. (Abs.at 660nm)	H_2 produced (ml H_2/ml -broth)	Substrate Hydrolysis (%)	pH	Cell conc. (Abs.at 660nm)	H_2 produced (ml H_2/ml -broth)	Substrate hydrolysis (%)
Glucose	6.26-6.3	3.64	0.363	49.1	6.07-6.21	1.90	0.595	45.2
Glycerol	7.89-8.19	1.35	0	ND	7.79	1.29	0.100	ND
Sucrose	7.47-7.61	1.83	0.140	ND	7.24	1.09	0.300	ND
Starch	7.44-7.53	0.81	0	<1.8	7.79	1.48	0.030	<1.5
Malate	7.51-7.75	3.10-3.36	1.590	>95	7.28-7.49	2.95	2.00-2.21	>95
Lactate	7.19-7.48	2.77	0.800	60.9	7.11-7.34	1.800	0.97-1.20	74.6
Acetate	9.22-9.43	3.10	0.235	55.2	7.37-7.57	1.375	0.370	15.5

Cells were grown in the 50 ml serum bottles using the modified Sistrom's broth at 30°C for 48 hr under 8 Klux/m^2 irradiance.
Initial pH of the culture was 6.8-7.0 and was not controlled during incubation.
Data were averaged from triplicated experiments.
All carbon sources were added 30 mM except starch (1%)

Two photo-bioreactors, A and B, of flat-rectangular shape were compared for the hydrogen production and β-D-HB accumulation in the culture broth during photo-incubation of *Rb. sphaeroides* KD131 *Hup*$^-$/*Phb*$^-$ mutant strain. Both of them had the same dimension, 20

cm × 20 cm × 6 cm (width × height × depth). Reactor A was made of glass for both wide sides and stainless steel for the edges, which was operated in the presence of cuprammonium rayon fibers inside under the photosynthetic experimental conditions. It caused the limited light illumination to the cells since the edges of the reactor were blocked from the light trasmittance and the fibers caused the shade in the reactor. However, the reactor B was made of clear plastic acryl material with 0.5cm thickness and all sides of the reactor were transparent and did not contain the cuprammonium rayon fibers. Hydrogen production was 820 ml H_2/L-broth for the first 17 hr of photo-incubation in the reactor A, while 1,200 ml H_2/L-broth was evolved in the reactor B under the same experimental conditions. At the same time, the amount of β-D-HB produced in the reactor A was approximately 1.8 times more than in the reactor B, and almost all of β-D-HB accumulated was degraded up to 55 hr of incubation during hydrogen production in the reactor B. β -D-HB produced in reactor A was also hydrolyzed for the rest of period of incubation but the rate of hydrogen production was 2 times lower than in the reactor B. The maximum β-D-HB accumulated in the reactor A for 24 hr was approximately 140-150 mM, which was determined by a calibration curve with standard β-D-HB using an Aminex HPX-87H organic acid analysis column. The concentration of β-D-HB may be over estimated because a unknown compound in D,L-malate and β-D-HB were eluted as the same peak during HPLC analysis. The calculation of D-β-HB concentration was assumed that this compound was not hydrolyzed at all at 24 hr of incubation.

Lee *at al.* [17] recently reported that the cellular PHB accumulated in the wild-type strain of *Rb. sphaeroides* KCTC 12085 up to 21.9 μg/mg dry weight of cells, while the mutant did not accumulate PHB during photo-incubation.

Imai *et al.* [19] studied PHB metabolism in *Rp. palustris* using NMR. They reported that PHB synthesis proceeded in the granules of the cells and under anaerobic light conditions, photo-metabolism and glycolysis generally competed with concomitant synthesis and decomposition of PHB, respectively, and that glycolysis gradually replaced photo-metabolism with aging of the cells. They also observed that photo-induced transport of β-D-HB through the membrane occurred when photo-metabolism and glycolysis were equally active in the light. However, it has been reported that *Rb. sphaeroides* OU 001 accumulated PHB 0.5g/L in the culture broth containing sugar refinery waste-water and malic acid as carbon sources.

The wild type and mutant strains produced 0.7-0.88 and 0.97-1.2 ml H_2/ml-broth containing 30 mM lactate as a carbon source, respectively and pH of culture broth increased to 7.3-7.4 for both strains. It showed the similar trend as in the cell growth using malate. Cell concentration of the mutant was 1.5 times less than the wild type strain but the amount of hydrogen produced by the mutant strain was 1.7 times more than the wild type strain when lactate was used as a carbon source. It seems that the mutant strain which was deleted the genes of PHB production and uptake hydrogenase, is more efficient for the hydrogen production than the wild type and it can be explained by two reasons. Firstly, the mutation deficient in both uptake hydrogenase and PHB formation had the positive effect on hydrogen accumulation. Secondly, the mutant strain always grew less cell density than the wild type strain during photo-incubation which resulted in the better light transmittance to produce the hydrogen efficiently.

When acetate was used as a carbon source, the initial pH 6.8-7.0 was increased to 9.2-9.4 by the wild-type strain but 7.4-7.6 by the mutant strain. The increase in pH by the wild-type strain might be due to the accumulation of PHB in cells. Khatipov *et al.*[11] also found that in nitrogen-deprived cells, the initial medium pH 7.5 increased to 10 and at the same time, PHB accumulation increased twice as compared to cells grown from an initial pH 6.8. Hydrogen production in this case decreased more than eightfold.

Wild type strain of *Rb.sphaeroides* KD 131 grew well in the Sistrom's broth containing

30 mM acetate as a carbon source to reach the cell concentration of absorbance 2.9-3.3 at 660 nm, and produced 0.2-0.3 ml H_2/ml-broth, while the cell growth of the mutant strain reached only 1.2-1.4 at 660nm under the same experimental condition and produced 0.35-0.39 ml H_2/ml-broth. Wild-type strain extensively accumulated the compound peaked at the elution time 27.16 min by HPLC chromatogram using an organic acid analysis column. We suspected this peak as a kind of oligomers of β-D-HB released from the cells because it was also shown in the HPLC chromatogram of the standard β-D, L-hydroxybutyrate as a contaminant at the same elution time. When hydrolysis of acetate increased, its content was increased with incubation time. However, the suspected oligomer accumulated in the culture broth did not degraded until 90-98 hr. The mutant strain did not accumulate the compound eluted at 27.16 min during photo-incubation when acetate was used as a carbon source, and β-D-HB did not accumulate in the culture broth for both strains.

The difference in cell growth of both strains in the culture media containing acetate as a carbon source might be due to the lack of PHB gene in the mutant strain because the wild-type strain of *Rp. sphaeroides* uses acetate as a carbon source to synthesize the cellular PHB in the light[20] while in the dark ^{13}C labeled acetate appeared first in C2 and C4 of butyrate and the subsequently in C4 of glutamate and C2 and C3 of succinate. Figure 1 shows the degradation of acetic acid by these strains, 46-50% acetic acid added was hydrolyzed in 24 hr by wild type strain, while only 25% was hydrolyzed in 72 hr of photo-incubation by the mutant.

Both of the *Rb. sphaeroides* wild-type and mutant strains did not grow well and did not produce hydrogen in the media containing glycerol or starch until 120 hr of photo-incubation. Glucose was a good carbon source for the cell growth of both strains for the first 48-55 hr of incubation until pH of culture broth is above 6.0. When glucose was hydrolyzed by *Rb. sphaeroides* to produce organic acids during incubation, pH decreased below 6.0 in both strains and cell growth stopped. The amount of hydrogen was 0.36 ml H_2/ml-broth for the wild type strain and 0.60 for the *Hup*$^-$/*Phb*$^-$ mutant strain, and only 45-49% glucose added was hydrolyzed.

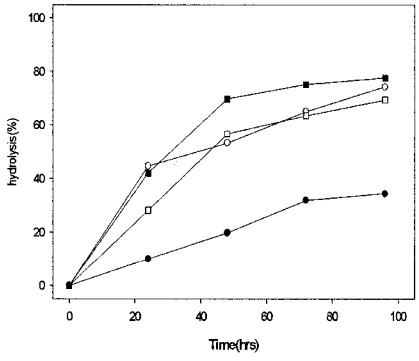

Figure 2. Hydrolysis of organic acids by *Rb. sphaeroides* KD131 wild type and mutant strains.
○, acetate /wild type ; ●, acetate /mutant ;
□, lactate /wild type ; ■, lactate /mutant

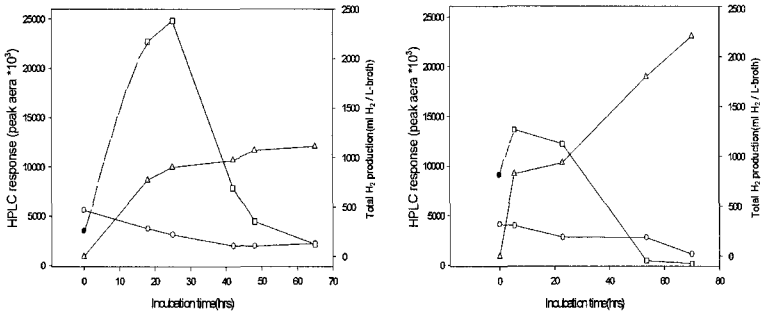

Figure 3. Changes of D,L- malate and D-β-hydroxybutyric acid contents in the culture broth of *Rb. sphaeroides* KD131 p-H- mutant during photo-incubation.

○, D,L-malate ; ●, impurity ; □, D-β-hydroxybutyrate ; △, hydrogen

A) Irradiance to a reactor was limited because the rectangular shape of the reactor contained cuprammonium rayon fiber and was made of glass for two flat sides and stainless steel for the edges.

B) Irradiance to a reactor was less limited because the rectangular shape of the reactor was made of clear acrylic glass.

Initial pH and Agitation

The cell growth of the *Rb. sphaeroides* KD131 wild type and mutant strains were better at the initial pH ranges of 6.8-7.2 compared to the other pH ranges of 5.0- 6.8 and 7.2-8.0 in the Sistrom's media containing D, L-malate and sodium glutamate. Hydrogen production by these strains was better at pH 7.4-7.6 than the rest of pH examined in the culture media. The cultures in this experiment clustered and floated after 2-3 days of photo-incubation even though they were agitated. It is not clear why the cells clustered in certain conditions, but it seemed to be related with the increase in pH and ion strength of broth during photo-incubation. The pH increase of the medium leads the dissipation of the cell membrane potential to cause the cells clustered. The clusters probably decreased the light efficiency of the cells to produce hydrogen. For the uniform irradiance and the increased light efficiency of cells, the cultures were stirred using a magnetic stirrer in small scale culture or an impeller in a flat-vertical type reactor during the photo-incubation, but hydrogen production not increased by agitation compared to the culture without agitation. The culture did not need continuous agitation to produce hydrogen from the *Rb. sphaeroides* KD 131 wild-type and mutant strains as long as the culture was stirred once in a while to prevent the precipitation at the bottom of reactor.

Light intensity

When the culture was illuminated at 7-8 klux/m^2 in one side of the surface of a reactor for 48 hr, the cell concentration of *Rb. sphaeroides* KD 131 wild type strain was as high as 2.9-3.4, absorbance at the 660 nm wavelength (Abs. 660 nm) and pH of culture broth was

7.4-7.5 using Sistrom's broth containing 30mM malate, while cultures without irradiance maintain the initial cell concentration 0.3-0.4 (Abs. 660nm) and pH 6.8-6.9. Irradiance of 110 Klux/m^2 decreased the cell growth and total hydrogen production for 48 hr, that is, approximately 50% of the cell concentration and 10-20% of the hydrogen produced at 7-8 Klux/m^2 were observed. When the culture was illuminated between 3 klux to 8 klux, cells grew well and showed red-brown color under a halogen lamp and green-brown color under a fluorescent lamp. However, when the light intensity was increased higher than 50 klux/m^2, cell growth slightly inhibited and the cultures turned gradually to pink-red color for 48 hr, and eventually lost the color to white over 120 hr of incubation. Whitening of the cells during photo-incubation was resulted due to the decrease in bacterio-chlorophyll contents of this strain as determined by absorbance at 854 nm. The spectral absorption of this strain was measured between the wavelength 200 nm and 1000 nm and exhibited two absorption maxima at 801 nm and 854 nm and a high amount of absorption at wavelength less than 500 nm. Losing bacterio-chlorophyll color of *Rhodobactor* strain during photo-incubation was reported by many researchers[21-24]. Tram- Werner *et al.*[22] reported that bleaching of purple bacteria *Rb. capsulata* occurred in the medium containing glucose both in the sun and at tungsten light, while experiment using lactate as a carbon source did not show the bleaching effect of cells under the same experimental condition. They suspected this phenomena that formate in the culture media, which was produced from glucose, was the cause of the pH drop in the culture resulting in the bleaching of cells. Others [21, 23, 24] also observed that *Rhodopseudomonas* strain was bleached at the outdoor cultivation when the irradiance of sun light at the surface of a reactor was higher than approximately 300-400 W/m^2. The bacterio-chlorophyll of the bleached cells were not restored by decreasing the light intensity. The mechanism of bleaching in purple bacteria is not completely understood. However, it is an important aspect to be understood in practical applications. The optimum light intensity for the hydrogen production and cell growth was 7-8 klux/m^2 for the wild-type strain of *Rb. sphaeroides* KD 131 under this experimental condition. Similar trends were observed in the mutant strain except that irradiance of 15-16 klux/m^2 was the optimum light intensity for the hydrogen production for 48 hr.

SUMMARY

1. A purple non-sulfur bacterium, *Rhodobacter sphaeroides* KD131 was isolated from nature and its mutants, *Hup*$^-$ and /or *Phb*$^-$ were derived by internal deletion of *Hup SL* and *Phb C*.
2. Maximum hydrogen was produced with *Hup*$^-$/*Phb*$^-$ mutant and the amount of hydrogen produced was in the increasing order of the wild type strain of *Rb. sphaeroides* KD131, *Phb*$^-$, *Hup*$^-$, *Hup*$^-$/*Phb*$^-$ mutants.
3. Hup$^-$/Phb$^-$ double mutant produced 2.2 ml H_2/ml-broth containing 30 mM malate and 8 mM L-glutamate at 30°C under 7-8 klux/m^2 irradiance using halogen lamps by illuminating at one side of a reactor.
4. Malate and lactate were better carbon sources for the hydrogen production than starch, sucrose and glycerol for the wild type and mutant strains.
5. When hydrogen production was limited, β-D-hydroxybutyrate accumulated in the culture broth for the first 20-25 hr of photo-incubation by both strains and then was hydrolyzed with time.
6. Acetic acid was hydrolyzed faster by the wild type strain than by mutant strain.
7. PH 6.8-7.2 was the optimum for the cell growth and pH 7.4-7.6 for the hydrogen production.
8. Irradiance of 7-16 klux/m^2 was the optimum for both cell growth and hydrogen production.
9. When the light intensity was increased higher than 50 klux/m^2, the cell growth was

slightly inhibited and the cultures turned gradually to pink-red color for the first 48 hr, and eventually lost its color to white over 120 hr of incubation.

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HYDROGEN PRODUCTION BY SUSPENSION AND IMMOBILIZED CULTURES OF PHOTOTROPHIC MICROORGANISMS. TECHNOLOGICAL ASPECTS.

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ABSTRACT

Hydrogen is recognized as the fuel of the future. Hydrogen can be produced by biological systems including purple bacteria, cyanobacteria, or microalga. Before practical consideration of photobiological hydrogen production many scientific and technological problems should be solved. Some of them are:

- Low efficiency of light energy bioconversion;
- Sensitivity of key enzymes (nitrogenase and hydrogenase) to oxygen;
- Low saturating light intensity comparing with sun light;
- Low specific rates of hydrogen photoproduction;
- Decrease of specific rates during scale-up procedure.

A numerous research articles using different approaches were published last decades. Simultaneously, the development of cultivation regimes, immobilization procedures, and photobioreactors bring some methods from the state-of-the art to the technology. This mini review is focused on some technological methods able to solve problems, shown above.

INTRODUCTION

In very near future the development of human population will be restricted by energy. Furthermore, it might happen not due to restrictions of fossil fuel but due to pollution of the nature by the products of its combustion. Molecular hydrogen is a valuable alternative to the fossil fuel since it may be generated from water, and water is the end product of its combustion. Several methods of H_2 production are under development with photobiological one being one of them. Phototrophic microorganisms are able for light-dependent hydrogen photoproduction under particular conditions. Purple bacteria and cyanobacteria produce H_2 under the light due to nitrogenase action whereas green microalga use hydrogenase.

The immediate application of phototrophic bacteria in biotechnological systems of H_2 generation is not possible due to many scientific and technological problems. Some of them

are:
- Low efficiency of light energy bioconversion;
- Sensitivity of key enzymes (nitrogenase and hydrogenase) to oxygen;
- Low saturating light intensity comparing with sun light;
- Low specific rates of hydrogen photoproduction;
- Decrease of specific rates during scale-up procedure.

To overcome these problems many molecular-biological, biochemical, physiological, and technological studies were done. This mini-review will discuss possible impact of technological methods to the solution of problems shown above in the case of nitrogenase-driven hydrogen production.

DEFINITIONS AND UNITS

Before the consideration of the potential of technological methods in improvement of biological hydrogen photoproduction some definitions should be done.

Several strategies for maximization of hydrogen photoproduction with different final targets are applicable.

1. Maximization of the hydrogen quantity
2. Maximization of the hydrogen production rate
3. Maximization of the time for stable hydrogen photoproduction.
4. Maximization of the efficiency of light energy utilization
5. Maximization of the efficiency of organic compounds utilization. This is the case for anoxygenic photosynthetic bacteria only.

For technological consideration the single strategy should be defined.

Maximization of hydrogen quantity without a consideration of time necessary for the process is not a practical strategy. In many cases the rate of hydrogen production as well as the efficiency of light energy bioconversion goes down with the time under unchanged conditions. With low rate of hydrogen production the system uses the occupied land inefficiently. Additionally, the cost of maintenance increases when time of operation is high.

Maximization of the hydrogen production rate leads to decrease of light and organic compounds conversion efficiencies [for reference see Tsygankov, 2001; Rocha et al.,, 2001 in the case of purple bacteria, and Hall, Rao, 1996; Pinto et al., 2002 in the case of cyanobacteria]. Vice versa, maximization of the efficiency of light energy utilization or organic compounds utilization results in low hydrogen production rate [see Akkerman et al., 2002; Koku et al., 2002]. However, all these parameters are important for practical system of light energy bioconversion.

One could suggest a kind of combination of strategies listed above: maximization of the steady-state rate of hydrogen production with usage of the sub-saturating light. If we get this target, we will get targets 1 and 3 simultaneously. In this case the system under optimal conditions of environment will be near the maximum efficiency of light energy conversion (due to the sub-saturating light), and not far from the maximum rate of hydrogen production. The application of steady state kinetics is important because in this case we could prolong the process as long as it is necessary. This kind of strategy suggests low efficiency of organic compound utilization by purple bacteria. So, it is applicable for the case when a source of organic compounds is wastewater and the system works as a pre-treatment element of wastewater purification. In following considerations this strategy will be considered.

Important question about units for hydrogen production rate arises. In the literature different units are used. They can be classified as specific rate per unit of culture, specific volumetric rates, and rates produced by one unit of illuminated surface.

Specific rate of H_2 production per unit of culture corresponds to the quantity of H_2 produced by the unit of culture in one unit of time. The unit of the culture might be expressed as the unit of the biomass dry weight [for example see Zurrer, Bachofen, 1982; Miyake, Kawamura, 1987; Tsygankov et al., 1998], the unit of the protein content [for example, see Tsygankov, Gogotov, 1982], the unit of chlorophyll [Hall, Rao, 1996]. Bacteriochlorophyll content, cells volume and quantity of cells might be also used as a measure of biomass quantity. Specific rate of H_2 production per culture is very important parameter. If the rate of H_2 production was measured under optimal conditions, it shows the potential of the strain. If this value was measured under particular conditions but for very well known strain, it characterizes the response of the culture to these conditions. This unit of measurements is of first importance during studies of environmental factors influence on hydrogen production or for comparative investigations of different strains. However, it does not show the possibility for hydrogen production by the strain when the culture is concentrated. It is well documented fact for purple bacteria and cyanobacteria that this rate goes down with the increase of cells concentration. So, this unit of measurements is not useful for estimation of actual rate of hydrogen production by particular photobioreactor.

H_2 production rate by one unit of illuminated surface is very useful for investigations of the efficiency of light energy bioconversion. For this case quantity of the biomass is not important. This specific rate must be distinguished from the rate of hydrogen production by the occupied surface. The illuminated surface might be much higher than occupied surface due to the configuration of a photobioreactor. For example, tubular photobioreactor occupied less than 0.25 m^2 and had an illuminated surface up to 0.65m^2[Tsygankov et al., 2002]. Furthermore, the illuminated surface might be increased without the increase of occupied land by increase of the height of the photobioreactor or by creation of sophisticated configuration. So, the rate of H_2 production by one unit of occupied surface does not give any information about the productivity of one unit of land occupied by the photobioreactor, its geometry or about biomass properties.

The rate of H_2 production by one unit of photobioreactor's volume is not useful for estimation of strain capabilities or for measurements of efficiency of light energy conversion. However, it is useful unit of measurements for optimization of hydrogen production by particular photobioreactor. From practical point of view it does not matter how much cells are in the photobioreactor or how much hydrogen is produced by one unit of illuminated surface. The rate of hydrogen production by the whole photobioreactor is of first importance. For a comparison of different photobioreactors it is better to express rate of hydrogen photoproduction per unit of its volume. So, it is practical unit for estimation of actual hydrogen photoproduction.

In our consideration we will use data from the literature where all three units are available for the comparative purposes.

SUSPENSION CULTURES

Cultivation regime

For investigations of microorganisms three regimes of cultivation with some modifications or combinations are used: batch cultures, fed-batch cultures, and continuous cultures.

Batch cultures are the cultures without an input of fresh medium and an output of cultural broth. Batch cultures are very simple in realization and widely used for estimation of the potential of different strains in hydrogen photoproduction or for study of environmental factors influence on the process [for reference, see Roha et al., 2001; Akkerman et al., 2002].

High rates of hydrogen photoproduction were recorded with application of this regime of cultivation (Tab. 1).

The main characteristic of batch cultures is that all the processes begin from the start of the culture and finish after substrate(s) consumption. So, hydrogen photoproduction by these cultures is accompanied by cells growth and possible simultaneous accumulation/degradation of storage polymers. That is why during estimation of efficiency of light energy bioconversion by batch cultures (and, especially, by resting cells) a special attention to the biopolymers quantity before and after experiments should be paid.

Fed-batch cultures differ from batch cultures by the possibility of additional input of the main substrate. Potentially, fed-batch cultures are very promising since in these cultures the possibility to prolong a hydrogen production phase with approximately constant rate exists. Unfortunately publications reporting the application of this cultivation regime for hydrogen production systems are not known to us.

Continuous cultures are the cultures with continuous medium input and culture output. When biomass concentration in photobioreactor is stable, rates of all processes as well as conditions for the culture are unchanged. This system is ideal for investigations of the culture response to changes of particular cultivation factor. Also it is the only system useful for the measurement and the optimization of efficiency of light energy bioconversion into biomass [Gobel, 1978; Pirt et al., 1980]. With application of continuous cultures optimal conditions for growth [Tsygankov, Gogotov, 1990] and nitrogenase activity [Tsygankov, Gogotov, 1982; Tsygankov et al., 1996] were established for purple bacterium *Rhodobacter capsulatus* B10.

Continuous cultures were used for hydrogen photoproduction studies by different purple bacteria (Tab. 2). Hydrogen photoproduction rates reported for continuous cultures are not the highest for purple bacteria (compare Tab. 1 and Tab. 2). Some reports on hydrogen photoproduction by batch cultures showed higher rates [Miyake, Kawamura, 1987; El-Shishtawy et al., 1997]. However, the processes were not steady state and data presented in Table 1 are maximum short-term rates in contrast to data in Table 2. That is why continuous cultures might be preferable for systems with the strategy suggested above. Using these cultures it is possible to solve some problems pointed in Introduction.

Table 1. Maximum hydrogen photoproduction by batch cultures or resting cells of purple bacteria

Strain	Conditions of cultivation	H_2 production			Reference
		$ml \cdot g^{-1} \cdot h^{-1}$	$ml \cdot cm^{-2} \cdot h^{-1}$	$ml \cdot l^{-1} \cdot h^{-1}$	
Rhodobacter sphaeroides RV	Supersaturating light, malate, acetate,butyrate, lactate; resting cells	262	0.790	262	Miyake, Kawamura, 1987
Rb. sphaeroides RV	Saturating light, lactate, glutamate; resting cells	180	0.116	235	Nakada et al., 1995
Rhodobacter sphaeroides S	Malate, glutamate, subsaturating light; batch culture	80.3	0.095	19	Sasaki, 1998
Rhodovulvum sp. H-1	Supersaturating light, malate, without N-source; resting cells	250	0.157	784	Yamada et al., 1998
Rhodobacter sphaeroides RV	Lactate, glutamate, saturating light; batch cultures	110*	0.758	81	El-Shishtawy et al., 1998

Only references with a possibility to calculate all specific rates of hydrogen production included here and in other tables.
* An approximation based on a general observation of final concentration of *Rb. sphaeroides* biomass grown in common medium with lactate [Miyake, Kawamura, 1987].

However, continuous suspension cultures have a drawback. Photosynthetic bacteria use light energy for maintenance and growth. Whereas maintenance expenditures for purple bacteria [Tsygankov, Laurinavichene, 1996] and cyanobacteria [Aiba, 1980] are small, expenditures for growth are high. For example continuous cultures of purple bacteria under optimal conditions for hydrogen production use 13-16 % of energy for hydrogen production and the rest – for biomass synthesis [Tsygankov et al., 1998]. Outdoor batch and continuous cultures of cyanobacteria use not more than 15 % of light energy for hydrogen production and the rest - for biomass synthesis [Tsygankov et al., 2002]. Since energy flow to biomass is proportional to the growth rate, it is important to keep cultures at the lowest possible growth rate. Exclusion of some substrate (nitrogen source for the case of purple bacteria) from the medium stops the growth. Unfortunately, in this case hydrogen photoproduction is not stable and goes down after several hours of incubation [Miyake, Kawamura, 1987]. Continuous suspension cultures of purple bacteria also have a limit of minimum growth rate. With decrease of growth rate below 0.04 h^{-1} nitrogenase activity of *Rb. capsulatus* B10 decreased [Tsygankov et al., 1998]. Cyanobacteria also show maximum nitrogenase activity in exponential growth phase [Tsygankov et al., 1997].

Photobioreactors

Many different types of photobioreactors for suspension cultures are described in literature. The photobioreactor construction should solve the main problem: an application of uniform light intensity to the culture. The detail description of photobioreactors is not a topic of present mini-review. For reference last reviews of photobioreactors are available [Tsygankov, 2001; Akkerman et al., 2002].

Here it is necessary to underline that no photobioreactor has a configuration appropriate for sufficient illumination of cultures with concentration more than 3-5 $g \cdot l^{-1}$.

Table 2. Maximum hydrogen photoproduction by continuous cultures of purple bacteria

Strain	Conditions of cultivation	H_2 production			Reference
		$ml \cdot g^{-1} \cdot h^{-1}$	$ml \cdot cm^{-2} \cdot h^{-1}$	$ml \cdot l^{-1} \cdot h^{-1}$	
Rhodospirillum rubrum S1	Chemostat with glutamate limitation, $D=0.013 \cdot h^{-1}$, lactate, light saturation	20	0.26	65	Zurrer, Bachofen, 1979
R. rubrum S1	Chemostat with glutamate limitation, lactate, light saturation; $D=0.01\text{-}0.05 \cdot h^{-1}$	100	0.515	170	Zurrer, Bachofen, 1982
Rhodobacter capsulatus B10	Chemostat wih ammonium limitation, lactate, continuous argon flow (100 ml/min), light saturation	88	0.115	97	Tsygankov et al., 1998

POSSIBILITIES OF TECHNOLOGICAL METHODS IN SOLUTION OF PROBLEMS RAISED IN BIOHYDROGEN PHOTOPRODUCTION RESEARCH

Low efficiency of light energy bioconversion

Using continuous cultures in appropriate photobioreactor with sub-saturating light intensities we could get the efficiency of light energy bioconversion near to the potential one (measured in short-term experiments under low light intensity). However, it is impossible to get the potential efficiency. For example, *Rb. sphaeroides* RV showed maximum efficiency of light energy bioconversion at 70 $W \cdot m^{-2}$ [Miyake, Kawamura, 1987]. Simultaneously, the rate of hydrogen production calculated per unit of surface was app. 25 % of maximum. At 250 $W \cdot m^{-2}$ (subsaturating light in this particular case) the rate of hydrogen production was 50 % of maximum but the efficiency decreased up to 50 % of the potential one. So, using our strategy we have to adopt that the steady-state rate of hydrogen production as well as the efficiency of light energy bioconversion under the optimal conditions might reach not more than 50 % of maximum values. We should expect only additional loss of efficiency with an increase of biomass concentration since in this case the culture will be under high gradient of light intensity which is not favorable for efficient light utilization [Tsygankov, Laurinavichene, 1996]. So, technological methods cannot improve the fundamental property of particular strain - the efficiency of light energy bioconversion. Basic research directed to the search of particular native strain or creation of specific mutant is necessary.

Sensitivity of key enzymes (nitrogenase and hydrogenase) to oxygen

This problem has a solution for batch and continuous suspension cultures of purple bacteria. Since purple bacteria do not need in gaseous substrate for hydrogen photoproduction, and do not produce oxygen, anoxic conditions could be reached by application of closed photobioreactor and degasation of input media. This problem is more difficult in the case of cyanobacteria. Simultaneously with hydrogen production they produce oxygen. Cyanobacteria developed several strategies for protection of nitrogenase, the key enzyme of hydrogen production, against toxic oxygen [Gallon, 1992]. As a result, nitrogenase activity of heterocystous cyanobacteria does not depend on oxygen up to as high as 50% of O_2 in gas phase even in mutant without hydrogenase [Tsygankov et al., 1998]. However, during photosynthesis O_2 concentration might be much higher. For that it is necessary to remove oxygen from photobioreactor. Air was used for exclusion of oxygen excess from photobioreactor with cyanobacteria [Tsygankov et al., 2002]. However, it is well known that molecular nitrogen has inhibitory effect on hydrogen production. The mixture of argon with limited concentrations of nitrogen was shown to be more efficient [Lichtl et al., 1997]. However, application of gas mixtures with nitrogen deficiency and hydrogenase inhibitors [Weissman, Benemann, 1977; Miyamoto et al., 1979] is expensive. Partial vacuum de-gasation also was used for removal of oxygen and hydrogen from cultures of immobilized cyanobacteria [Hall, Rao, 1996]. Technology of gas separation also is a possibility to overcome the problem [Teplyakov et al., 2002].

Low saturating light intensity comparing with sun light

Several approaches, including molecular biological, to raise the light intensity saturating threshold are under investigation [Hallenbeck, Benemann, 2002]. One of them is to find or develop mutants of photosynthetic microorganisms with reduced size of light harvesting complexes with unchanged quantity of reaction centers [Vasilieva, 1998; Melis; 2002].

Recently, Tredici and Zitelly [1998] suggested technological method of "spatial dilution of light". This method might be illustrated as follows. If we take a thin rectangle cuvette as a photobioreactor and put it under the sun with the surface perpendicular to sun beams, the culture will be exposed to sun light intensity. However, if we decline the cuvette with the angle α from sun beams, the culture will be exposed to the intensity which is lower than direct sun by factor $\sin\alpha$. Using this method, it is possible to keep optimal (subsaturating in our case) light intensity by simple inclination of photobioreactor. This way it is possible "dilute" sun light as much as necessary in photobioreactor with any configuration. So, the problem of low saturating light intensity has technological solution.

Low specific rates of hydrogen photoproduction

The specific volumetric rate of hydrogen photoproduction depends on the rate of hydrogen production by one unit of biomass and on the concentration of the biomass in a photobioreactor. The improvement of a biomass activity is the basic problem for strain selection or construction. The specific volumetric rate might be increased technologically by increase of biomass concentration.

Real biomass concentration in laboratory scale photobioreactors usually is in the range 1.5-5 g dry weight$\cdot l^{-1}$ [for reference see Rocha et al., 2001]. For comparison, intensive cultures of chemotrophic microorganisms in laboratory experiments produce cultures with concentration 138 g$\cdot l^{-1}$ for *Candida brassicae* [Suzuki et al., 1985]; up to 145 g dry weight$\cdot l^{-1}$ for *Escherichia coli* [Lee et al., 1990]. Industrial fermenters for yeasts usually designed for 15-35 g$\cdot l^{-1}$ of dry weight [Viesturs et al., 1986]. Fixed-bed reactor could contain sludge with concentration of solids up to 65 g$\cdot l^{-1}$ [Chang et al., 2002]. So, if we are able to use concentrated biomass of photosynthetic microorganims without the loss of activity, we'll have the way to enhance specific volumetric rate of hydrogen photoproduction. Unfortunately, the decrease of specific microbial activity with increase of biomass concentration is well documented fact for photosynthetic microorganisms. In suspension cultures even at 1 g$\cdot l^{-1}$ of biomass the specific biomass activity decreased in many photobioreactors [Zurrer, Bachofen, 1982; Tsygankov et al., 1998]. It is because of exponential decay of light intensity in light absorbing media. Technological solution based on rapid mixing of cells was shown to be impractical [Hallenbeck, Benemann, 2002]. Another solution of the problem for suspension cultures lies in decrease of suspension depth for light penetration [Ogbonna et al., 2001; Akkerman et al., 2002]. However, the construction of photobioreactors with a thickness less than 2-3 mm is very difficult. In thin layers the problem of homogeneity of suspensions arises. Additionally, cells create a film on transparent walls of photobioreactors, which prevents light penetration into the suspension. So, suspension cultures have upper limit of cell concentration with efficient light energy utilization.

From technological point of view using suspension cultures we put cells into the medium and trying to bring them, together with a medium, to the light. In this case a bacterial film on walls or clot formation are declared as waste processes. However, we can change our culture control : put them to the light and bring to them (remove from them) substrates (products) with medium which is free of cells. In this case active biofilms as well as clots are useful and we get immobilized cultures.

IMMOBILIZED CULTURES

Methods and matrixes for immobilization of photosynthetic microorganisms

Immobilized cells are widely used for both practical and academic purposes. Immobilization techniques have been developed for cell stabilization and easy operation. The methods for immobilizing of the whole cells may be categorized as follows:

- entrapment by inert support like gel;
- adsorption by an inert support like ion exchange material;
- binding via immobilized biological macromolecules or attachment to the activated surface of the matrix
- autoimmobilization.

Different kinds of gels were used for cell entrapment [Brodelius, Vandamme, 1987]. Photosynthetic microorganisms were entrapped into translucent gels like agar [Vincenzini et al., 1981], carrageenan [Francou, Vignais, 1984], poly(vinil alcohol), and alginate [Hallenbeck, 1983]. Polyacrylamide inactivated purple bacteria [Weetal, Krampus, 1980], whereas agarose was shown to be unstable during operation [von Felten et al., 1985].

Immobilization of microorganisms in gels has a drawback. High concentration of cells can give high rates of substrate consumption and product formation. In some cases these rates are limited not by light but by substrate diffusion through gel matrix [Vincenzini et al., 1982] or through composite agar/microporous membrane structure [Planchard et al., 1989]. Diffusion and reaction rates through gel membrane were quantitatively described earlier [Backer et al., 1992]. To overcome this drawback another methods of immobilization with a possibility of direct contact of microorganisms with medium were explored.

Immobilization on solid matrix by adsorption on ion exchange material or binding via lecitin is widely used for chemotrophic bacteria [Jack, Zajic, 1977] but not reported for photosynthetic microorganisms. In these matrixes a direct contact of microorganisms with medium was possible. However, matrixes and ion exchange material for immobilization of photosynthetic microorganisms should be transparent or translucent.

Translucent porous glass matrix was used for immobilization of purple bacteria [Tsygankov et al., 1994]. To accelerate an attachment of purple bacteria to glass surface the method of matrix activation by 3-(2-aminoethyl-aminopropyl)-trimethoxysilane [Tsygankov et al., 1993]. After this activation glass surface reacted with environment by positively charged aminoresidues. As a result photosynthetic microorganisms covered 2-38 % of activated glass surface after 1 h of incubation of microbial suspension in distilled water with glass matrix [Tsygankov et al., 1998].

Quick immobilization was suggested for cyanobacteria. It was possible to polymerize polyurethane foam together with microalga or cyanobacteria without essential loss of activity [Brouers et al., 1983].

Immobilization of microorganisms on activated surfaces or polymerization of foam with microorganisms is quick but needs careful handling and expensive reagents. Autoimmobilization is cheaper. The time of autoimmobilization depends on the strain origin and matrix properties, varies from days to several weeks [Hall, Rao, 1989; Tramm-Werner et al, 1996] and starts from biofilm formation. Biofilm formation includes several initial steps [Bos et al., 1999]. When microorganisms and matrix are in an aqueous environment, matrix surface will first become covered with a layer of adsorbed, organic molecules generally called 'conditioning film'. Transport of microorganisms, as the second step of biofilm formation, may include Brownian motion, gravitation, diffusion or the motility of microorganisms. Simultaneously microbial coaggregates can be formed. Subsequently, microbial adhesion occurs. Initially the adhesion is reversible and becomes irreversible in time due to excretion of exopolymers

by the adhering microorganisms. Single adhering cells stimulate the adhesion of others through phenomenon known as 'co-adhesion'. Eventually, adhering microorganisms grow.

This is the major factor contributing to the accumulation of a high number of attached cells. After biofilm formation the biomass growth depends mostly on the medium content and might be as quick as the growth rate of microorganisms.

Application of immobilization technique and designing matrix with thin layer gives an opportunity to reach high cell density in one unit of volume. Thin layer allows keeping the optimal concentration of cells per unit of illuminated surface even with high density of culture. For example, in 13 mm thick photobioreactor with suspensions of *Rb capsulatus* the optimal concentration for hydrogen photoproduction was app. $1.5 g \cdot l^{-1}$ [Tsygankov et al., 1998]. It corresponds to $1.15 g \cdot cm^{-2}$ of biomass surface concentration. With a thickness 0.5 mm of immobilized cells [as in the case of porous glass sheet, Tsygankov et al., 1994], the same concentration of cells per unit of illuminated surface corresponds to $39 g \cdot l^{-1}$ of volumetric biomass concentration.

Matrix material is very important for the time of biofilm formation. Clean glass surfaces are repulsive for microorganisms [Tsygankov et al., 1993], and biofilm formation is very long. The influence of matrix material, roughness of the surface, flow velocity, culture age and light intensity on the biofilm formation by *Rb. capsulatus* was studied [Tramm-Werner et al., 1996]. After 24 h of incubation polycarbonate, polyamide, and polybutylic terephtalate accumulated 0.1-0.3 g of biofilm dry matter per cm^2 of matrix. However, polyethylene and polypropylene did not accumulate any biofilm during this period. The surface roughness in the range 0-5 μm did not influence the biofilm formation. Light intensity during cells incubation was shown to be important factor for biofilm formation. Cultures from late stationary phase (older than 13 days) did not produce biofilms.

Other matrixes, for example glass beads [Smith, Lambert, 1981], hollow fibers [Wang et al., 1991] were also used for immobilization of cyanobacteria. However, evidences for advantage of these matrixes are not satisfactory.

Hydrogen production by immobilized cultures

Some literature data have allowed us to calculate three types of hydrogen production rates (Tab. 3) and compare them with data for suspension cultures.

Efficiency of light energy bioconversion was calculated as the ratio between an energy accumulated in H_2 produced by 1 cm^2 of surface and an incident light energy.

Cells immobilized in gels showed lower volumetric rates of hydrogen photoproduction as compared with immobilization on porous glass. In some cases, the diffusion limitation of the process rate was the main reason as shown above. In other cases thick layer of dense culture was insufficiently illuminated. The observation, that hydrogen production rate was higher when the layer of matrix was thinner, supports this suggestion (Tab. 3).

Planchard et al. [1989] has reported much higher rate of hydrogen production by 1 g of immobilized biomass than in resting cells or batch cultures [Planchard et al., 1989]. It should be noted, however, that this value, based on initial biomass concentration, might be not valid due to possible growth of microorganisms in a matrix [von Felten et al., 1985; Fedorov et al., 1999].

Immobilized cultures even without continuous medium flow showed remarkably higher stability than batch cultures or resting cells. Application of continuous medium flow resulted in stable operation up to 3600 h [von Felten et al., 1985]. In general, bacteria immobilized on thin matrixes provided highest volumetric rates of hydrogen photoproduction. Here is a key for intensification of the process using thin plates with high concentration of biomass [Tsygankov, 2001b].

Table 3. Maximum hydrogen production rates by immobilized cultures of purple bacteria

Strain and conditions	Efficiency of light energy bioconversion, %	H_2 production			Reference
		$ml \cdot g^{-1} \cdot h^{-1}$	$ml \cdot cm^{-2} \cdot h^{-1}$	$ml \cdot l^{-1} \cdot h^{-1}$	
Rps. palustris 42OL, entrapped into agar gel, malate,glutamate, yeast extract, over 30 days of semi-continuous operation; 69.5 $cal \cdot h^{-1} \cdot 100\ cm^{-2}$	1.2	40	0.0178	44	Vincenzini et al., 1981
R. rubrum, agar beads, lactate, glutamate, continuous medium flow, , more than 3000 h of operation; 1300 $W \cdot m^{-2}$	-	50	-	57.3	Von Felten et al., 1985
R. rubrum PCC7061, composite agar gel and microporous membrane structure; malate, glutamate; up to 83 h of batch operation; 15 klx	-	565	0.169	565	Planchard et al., 1989
a) *Rb. sphaeroides* RV, matrix -porous glass (0.5 mm), succinate, glutamate, continuous medium flow; 11.2 mg dry weight per ml	2.10	116	0.027	560	Tsygankov et al., 1994
of matrix; 40 $W \cdot m^{-2}$; 900 h of operation					
b) 300 $W \cdot m^{-2}$	0.67	-	0.065	310	
a) *Rb. sphaeroides* GL1, matrix - polyurethane foam (5 mm thickness), succinate, glutamate, continuous medium flow; 120 $W \cdot m^{-2}$; 500 h of operation	0.76		0.076	210	Fedorov et al., 1999
a) *Rb. sphaeroides* GL1, matrix - porous glass, succinate, glutamate, continuous medium flow; 120 $W \cdot m^{-2}$; 850 h of operation	2.22	-	0.089	1780	Tsygankov et al., 1998
b) –"- 300 $W \cdot m^{-2}$	1.90	-	0.19	3800	-"-
c) *R. fulvum* K5, waste water from milk factory, continuous flow; 30 $W \cdot m^{-2}$; 750 h of operation	1.30	-	0.013	260	-"-

Comparing with resting cells, batch and continuous cultures, the immobilized systems showed decreased rates of hydrogen photoproduction per unit of surface. As a result the efficiency of hydrogen photoproduction by these systems were also lower than highest reported for resting cells [Miyake, Kawamura, 1987]. It suggests that cultures had not optimal conditions in some respect. So, here is a room for further improvement of systems with immobilized purple bacteria.

Immobilization of purple bacteria on porous glass has advantage, which is not applicable for another matrixes. Here is a possibility of illumination from thin side with high hydrogen evolution rate, which did not saturated even at 5000 $W \cdot m^{-2}$ (Fig. 1). So, even with a surface parallel to sun beams the illumination of cells on porous glass is possible. It gives a good opportunity for scaling up procedure [Tsygankov 2001]. Also, this is the example how technology could solve the problem called as "low saturation light intensity" (see Introduction)

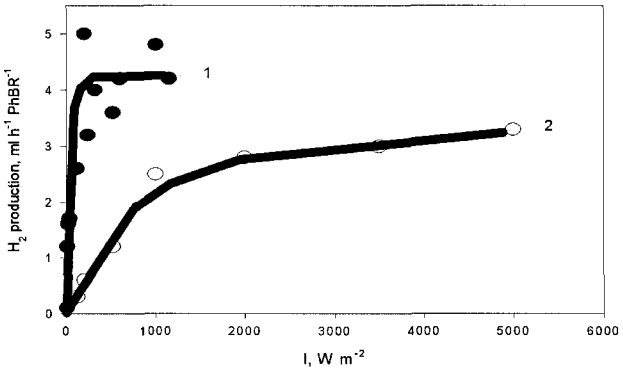

Figure 1. The rate of hydrogen photoproduction as a function of incident ligh intensity. Photobioreactor [Tsygankov et al., 1994] with *Rb. sphaeroides* RV immobilised on porous glass ($125 \times 50 \times 0.5$ mm^3) with continuous medium flow ($5\ ml \cdot h^{-1} \cdot reactor^{-1}$) was illuminated from face (thickness 0.5 mm; curve 1) or side position (thickness 50 mm; curve 2) by halogen lamp [Tsygankov, Miyake, unpublished].

In conclusion, technological methods such as continuous medium flow, immobilized cells, and photobioreactor with particular illumination design have high potential in solution of following problems:

- Sensitivity of key enzymes (nitrogenase and hydrogenase) to oxygen;
- Low saturating light intensity comparing with sun light;
- Low specific rates of hydrogen photoproduction;
- Decrease of specific rates during scale-up procedure.

Thus, if the problem of low efficiency of light energy bioconversion will be solved, technological research with clean strategy and evident goals will produce real practical system in very near future after. However, even in this case, much experimental research should be done to optimize the whole system.

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III. Hydrogenase

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THE POTENTIAL OF USING CYANOBACTERIA AS PRODUCERS OF MOLECULAR HYDROGEN

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INTRODUCTION

Molecular hydrogen (H_2) is a future energy carrier that may be a valuable alternative to the limited fossil fuel resources of today-it is a most relevant solution in the future for mankind. Used in e.g. fuel cells it generates electricity and will drive cars, buses as well as heat our houses. Today H_2 is produced from either fossil fuels or from water via electrolysis. The choice of method is based on the relative prices of the systems. H_2 can also be produced from renewable sources e.g. chemoorganootrophically from biomass and phototrophically using microorganisms. An attractive possibility is the direct splitting of water for generation of H_2 using solar radiation. This splitting can be achieved in photochemical fuel cells, and by applying photovoltaics which directly utilizes solar radiation for electrolysis of water into H_2 and O_2. The most challenging option is the production of hydrogen by photosynthetic microorganisms. For photobiological H_2 production, cyanobacteria are among the ideal candidates since they have minimal nutritional requirements: They can thrive on air ($N_2$and CO_2), water (electrons and reductant), and mineral salts with light as the only energy source.

CYANOBACTERIAL HYDROGENASES

Cyanobacteria may possess several enzymes directly involved in H_2 metabolism: nitrogenase(s) catalyzing the production of H_2 concomitantly with the reduction of nitrogen to ammonia, an uptake hydrogenase, catalyzing the consumption of H_2 produced by the nitrogenase, and a bidirectional hydrogenase, which has the capacity to both take up and produce H_2 [for a recent review see Tamagnini et al 2002].

An uptake hydrogenase, with the evident function of catalyzing the consumption of the hydrogen produced by nitrogenase, has been found in all N_2-fixing cyanobacteria examined so far. This enzyme appears to be membrane-bound and, in some filamentous strains, is particularly expressed in the N_2-fixing heterocysts with little or no activity in the photosynthetic vegetative cells. A strong correlation between the activity of an uptake hydrogenase and N_2-fixation has been demonstrated in filamentous cyanobacteria.

The first molecular data concerning cyanobacterial hydrogenases appeared in 1995, a

novel developmental genome rearrangement for *Anabaena* PCC 7120 during the differentiation of a vegetative cell into a heterocyst [Carrasco et al 1995] This rearrangement occurs within a gene (*hupL*) encoding the large subunit of the uptake hydrogenase. The excision of a 10.5 kb DNA element occurs late during the heterocyst differentiation process indicating that *hupL*, in *Anabaena* PCC 7120, is expressed in heterocysts only. The excision occurs by a site-specific recombination, and the recombinase gene, *xisC*, was found 115 bp inside the right border of the excised element. Subsequently, the structural genes encoding both the small (*hupS*) and the large (*hupL*) subunit of the uptake hydrogenase have been sequenced and characterized in *Anabaena* PCC 7120, *Nostoc* PCC 73102 [Oxelfelt et al 1998], and in *A. variabilis* [Happe et al 2000]. All cyanobacterial *hupSL* genes sequenced so far are highly conserved, ranging from 83.8 % to 95.1% nucleotide identities, except for the fact that the occurrence of the *hupL* rearrangement/presence of *xisC* is not ubiquitous. They do, however, collectively differ significantly from the corresponding genes in other microorganisms. The deduced amino acid sequences of the cyanobacterial uptake hydrogenases all have >93% similarities when compared to each other. Generally, the structural genes encoding NiFe-hydrogenases are clustered in a similar physical organization forming a transcript unit, *hupS* being located upstream of *hupL*. In several microorganisms, a third ORF, *hupC*, has been identified and is located directly downstream of the *hupSL* genes. It has been proposed that *hupC*, a b-type cytochrome, could play a role in mediating the electron transport to the terminal acceptor oxygen. The cyanobacterial *hupSL* genes follow the general pattern, and are transcribed as a single transcript in *A. variabilis* and in *Nostoc* PCC 73102. In both organisms, as well as in *Anabaena* PCC 7120, ORFs have been detected downstream of the respective *hupL* genes but none of them show any similarity to *hupC*.

The first transcriptional study concerning cyanobacterial *hupSL* genes was performed with *Anabaena* PCC 7120 where non-N_2-fixing filaments of vegetative cells were transferred to N_2-fixing conditions [Carasco et al 1995]. RT-PCR demonstrated that *hupL* transcription coincides with heterocyst formation. Subsequent studies of *N. muscorum* [Axelsson et al 1999], *Nostoc* PCC 73102 [Hansel et al 2001] and *A. variabilis* [Happe et al 2000], using RT-PCR and Northern blot, respectively, confirmed the induction of a *hupL* transcript under N_2-fixing conditions only. In *N. muscorum*, the induction of the transcript was followed by the appearance of an *in vivo* hydrogen uptake activity.

The *hupSL* transcript in *A. variabilis* is 2.7 kb, and the transcription start site is located 103 bp upstream of the start codon, whereas in *Nostoc* PCC 73102 it is situated 259 bp upstream. Both start sites are preceded by putative -10 sequences and putative promoter elements have been identified. In the *A. variabilis* sequence, it is possible to identify one half of a consensus Fnr-binding sequence, and in *Nostoc* PCC 73102 there are possible IHF (Integration Host Factor) and NtcA binding sites. An Fnr (Fumarate nitrate reductase regulator) binding site is involved in the regulation of the *E. coli hyp* operon, and the protein induces several operons during anaerobic growth. IHF is known to be involved in transcriptional activation of *nif* genes in purple bacteria, and the global cyanobacterial nitrogen regulator NtcA has been shown to be necessary for heterocyst differentiation and expression of some of the genes involved in N_2-fixation in *Anabaena* PCC 7120.

The cyanobacterial genes differ from *hupSL* of other microorganisms in being separated by longer intergenic regions. These regions have been found to consist largely of short tandemly repeated repetitive sequences (STRRs). Even though the specific STRRs are not

conserved, sequence analyzes revealed that the possibility of a hairpin formation is a common feature indicating a conserved two-dimensional structure rather than a specific sequence of the repeat itself. The specific function(s) and origin of the repeats are not known. One possible function of hairpins could be to increase the stability of the transcript. Another suggestion is that the hairpin structure may confer a translational coupling between the two structural genes.

The soluble or loosely membrane associated cyanobacterial bidirectional hydrogenase is a common enzyme in both N_2-fixing and non-N_2-fixing cyanobacteria. However, recent data clearly demonstrated that, at least in N_2-fixing strains, it is not a universal enzyme and that strains missing the bidirectional enzyme have nothing obvious in common [Tamagnini et al 2000]. The physiological role of the bidirectional hydrogenase has been a matter of speculation, and is still unclear. One possibility is that it functions as a mean of releasing excess of reducing power in anaerobic environments and as discussed below in (at least in) some strains it performs a function in cyanobacterial fermentation [Troshina et al 2002].

In 1995, a set of structural genes (*hox* genes) encoding a bidirectional hydrogenase in *A. variabilis* was sequenced and the bidirectional enzyme was suggested to be a heterotetrameric enzyme consisting of a hydrogenase part (encoded by *hoxYH*) and a diaphorase part (encoded by *hoxFU*) [Schmitz et al 1995] The presence of third diaphorase subunit (*hoxE*) has been clearly demonstrated for *Anacystis nidulans* (=*Synechococcus* PCC 6301) and *Synechocystis* PCC 6803 [Schmitz et al 2002]. In subsequent works, *hox* genes have been sequenced and characterized in the unicellular *Synechocystis* PCC 6803, *Synechococcus* PCC 6301, and (partially) in *Chroococcidiopsis thermalis* CALU 758, as well as in the filamentous strains *Anabaena* PCC 7120 and (partially) in *A. variabilis* IAM M58. The physical organization of the structural genes encoding the bidirectional enzyme is similar. In *A. variabilis*, *Anabaena* PCC 7120, *Synechococcus* PCC 6301, and *Synechocystis* PCC 6803, one or several additional ORFs have been identified between some of the structural genes. In *A. variabilis* IAM M58, an additional ORF is localized between *hoxF* and *hoxU*.

Nucleotide sequence comparisons have shown that there is a reasonably high degree of homology between the *hox* genes of cyanobacteria and genes encoding the NAD^+-reducing hydrogenase from e.g. the chemo-lithotrophic hydrogen metabolizing bacterium *Ralstonia eutropha*. Sequence comparisons between the bidirectional hydrogenase genes/deduced proteins of *A. variabilis* ATCC 29413, *A. variabilis* IAM M58, *Anabaena* PCC 7120, *Synechococcus* PCC 6301, and *Synechocystis* PCC 6803 reveal that they are highly homologous, with *A. variabilis* ATCC 29413 and *Anabaena* PCC 7120 being at least 95 % identical when comparing the deduced amino acid proteins. The two *A. variabilis* strains are less similar (78-88 % identical), and all other comparisons are in the range of 58 to 71 % identical deduced proteins.

Transcriptional studies (using RT-PCR) indicate that the structural genes form a single transcriptional unit in *A. variabilis* [see Tamagnini et al 2002]. In contrast, in the unicellular *Synechococcus* PCC 6301 two transcripts have been detected; the dicistronic *hoxEF*, and the polycistronic *hoxUYHWhypAB*. Further promoter activities were identified in the *hox* locus using β-galactosidase and bacterial luciferase as reporters. In a shift from non-N_2-fixing to N_2-fixing conditions, no significant change in the transcript level of *hoxH* in *N. muscorum* was observed.

ACCESSORY GENES & GENE PRODUCTS

The maturation of nickel-containing enzymes, e.g. hydrogenases, ureases, and carbon monoxide dehydrogenases, is a complex process requiring accessory proteins. Initial work using *Escherichia coli* revealed five ORFs, designated *hypABCDE,* affecting hydrogenases pleiotropically. Although hydrogenase genes have been identified and characterized in several cyanobacteria, very little is known about the cyanobacterial *hyp* genes and/or corresponding *hyp* proteins [for a summary see Tamagnini et al 2002]. The first *hyp* genes to be identified were *hypABF* in *Synechococcus* PCC 6301 and *hypB* from *Anabaena* PCC 7120. The former are preceded by structural genes encoding the bidirectional hydrogenase (*hoxUYH*) and the putative protease *hoxW*, which might be involved in the maturation of *hoxH*. Transcriptional analysis (RT-PCR) revealed that *hypA* and *hypB* form a polycistronic transcript together with *hoxUYHW*, whereas *hypF* is part of a different transcript. *hypB* of *Anabaena* PCC 7120 is part of a gene cluster consisting of *hypBAEDF*, *hypA* and *hypB* overlapping by 10 bp. A *hyp* gene cluster consisting of *hypFCDEAB* and an additional upstream ORF has been cloned and sequenced from *Nostoc* PCC 73102 [Hansel et al 2001]. In *Synechocystis* PCC 6803, the *hyp* genes are scattered over the genome, with the exception of *hypA'* and *hypB'* which have the same direction and are only 61 bp apart.

DIVERSITY OF CYANOBACTERIAL HYDROGENASES

The molecular diversity of cyanobacterial hydrogenases has been examined in a selected set of N_2-fixing *Anabaena* and *Nostoc* strains [Tamagnini et al 2000, Tamagnini et al 2002]. Although *hup* gene homologues are present in all strains examined, all kinds of combinations can be detected concerning the presence/absence of sequences homologous to *xisC*, and to the bidirectional hydrogenase genes. Interestingly, at present there are no data supporting the existence of either multiple uptake hydrogenases or multiple bidirectional hydrogenases in a single cyanobacterial strain. There is no obvious connection to the different habitats from where the individual strains originally were isolated. Interestingly, *Anabaena* PCC 7120 contains only one set of *hyp* genes, and since both an uptake and a bidirectional hydrogenase are present in this strain it suggests that one copy is enough to fulfill the functions for both hydrogenases. Assuming this to be a general feature for cyanobacteria, the specific regulation of *hyp* genes in response to the differentially regulated hydrogenases in a single cyanobacterial strain deserves future attention.

CYANOBACTERIAL BIOHYDROGEN

Basically, two strategies are possible for cyanobacterial biohydrogen: (1) H_2 produced by nitrogenase, and (2) H_2 produced by the bidirectional hydrogenase. Individual strains may harbor both a bidirectional hydrogenase (though this is not a universal cyanobacterial enzyme), and none to several nitrogenase(s), encoded by different structural genes. There is no report describing the existence of either several uptake hydrogenases or several bidirectional hydrogenases in a single cyanobacterial strain. An efficient photoconversion of water to hydrogen by cyanobacteria is certainly influenced by many other factors, and only an extensive knowledge within this field can lead to the improvement of the rates of cyanobacterial hydrogen production. For example, it is known that immobilized cells produce more hydrogen than free-living cultures, and that non-Mo-containing nitrogenases allocate more electrons to the production of hydrogen. The gas phase, the age and density of the

culture, as well as the composition, pH, and temperature of the growth medium are also crucial for the final result. Most of the research on cyanobacterial hydrogen production has been carried out with nitrogen-fixing strains. In these organisms, the net hydrogen production is the result of hydrogen evolution catalyzed by nitrogenase and hydrogen consumption mainly catalyzed by an uptake hydrogenase. Consequently, the production and selection of mutants deficient in hydrogen uptake activity is of great interest. Moreover, the nitrogenase has a high ATP requirement and this lowers considerably their potential solar energy conversion efficiencies. On the other hand, the bidirectional hydrogenase requires much less metabolic energy, but it is very sensitive to oxygen.

Most of the research on rates of cyanobacterial hydrogen production has been carried out with nitrogen-fixing filamentous strains with typical values ranging from 0.17 to 4.2 nmol of hydrogen produced per μg chlorophyll *a* and hour [Tamagnini et al 2002]. Interestingly, nitrogen-fixing cells of *Anabaena variabilis* SPU 003 have been shown to posses the capacity to produce hydrogen mainly in darkness. Addition of various sugars stimulated the production of hydrogen with mannose giving the highest rate, 5.6 nmol hydrogen produced per mg dry weight and hour. Since the hydrogen production is the result of hydrogen evolution catalyzed by nitrogenase and a consumption of hydrogen mainly catalyzed by an uptake hydrogenase the obvious improvements are to increase the hydrogen production by using alternative nitrogenases and by inhibiting the activity of the uptake hydrogenase. In a recent study, the hydrogen production by autotrophic, vanadium-grown cells (i.e. cells expressing the alternative vanadium-containing nitrogenase) of *A. variabilis* ATCC 29413 (wildtype) and of its mutant PK84 impaired in the utilization of molecular hydrogen, was studied in a photobioreactor. The highest hydrogen production rates were observed in cultures grown at gradually increased irradiation. The wildtype strain evolved hydrogen only under argon atmosphere with the actual rate being as high as the potential rate (61 % of oxygenic photosynthesis used for hydrogen production). In contrast, cells of PK84 also produced hydrogen during growth under carbon dioxide enriched air (13 % of oxygenic photosynthesis used for hydrogen production representing 33 % of the potential rate). *A. variabilis* ATCC 29413 PK84 has also been examined under simulated outdoor conditions. Using a 4.35 liter automated helical tubular bioreactor and continuous cultivation for 2.5 months resulted in a maximum hydrogen evolution of 230 ml hydrogen produced per 12 h light period at a growth density of 3.6 to 4.6 μg chlorophyll *a* per ml cell culture. Replacing air with argon doubled the rate of hydrogen evolution. Anoxygenic conditions over the dark periods had a negative effect on hydrogen production. Similarly, in aerobic outdoor conditions operated for 4 months a maximum rate of 80 ml hydrogen per reactor (4.35 L) and hour was obtained on a bright day from a batch culture. The maximum efficiency of conversion of light to chemical energy of hydrogen was calculated to be 0.33 % and 0.14 % on a cloudy and a sunny day, respectively.

Nostoc PCC 73102 has become a model strain for cyanobacterial research. The genome is known [see Tamagnini et al 2002] and genetics, including the generation of mutants, is possible. *Nostoc* PCC 73102 contains one nitrogenase, one uptake hydrogenase [Oxelfelt et al 1998] and no bidirectional hydrogenase [Tamagnini et al 1997]. A genetically engineered *hup* minus mutant constantly produces molecular hydrogen through the action of nitrogenase and the produced H_2 rapidly leaves the cell suspension [Lindberg et al 2002]. This hydrogenase free cyanobacterium, which originally (before it was isolated and brought into a free-living condition) functioned *in situ* as a natural bioreactor in coralloid roots of cycads, may become an interesting "starting strain" when designing future cyanobacteria with both improved hydrogen production and evolution as well as reduced growth rate.

In a recent study, the filamentous cyanobacterium *Anabaena* PCC 7120 (wildtype) containing one nitrogenase, one uptake hydrogenase and one bidirectional hydrogenase and its hydrogen uptake deficient mutant AMC414 were analysed for their H_2 production capacities [Lindblad et al 2002]. *Anabaena* PCC 7120 and AMC414 had similar growth rates in turbidostat mode with increased growth rates at higher light intensity. Rates of C_2H_2 reduction were similar for both strains. In contrast to the wildtype, AMC414 produced H_2 in a PhotoBioReactor (PhBR) using air as the lifting gas. The rate of H_2 production increased with light intensity and was not even saturated at 456 $\mu E \cdot m^{-2} \cdot s^{-1}$. H_2 production increased significantly when replacing the air with argon. The maximal H_2 production during outdoor conditions was recorded using AMC414 with a peak at 14.9 ml $H_2 \cdot h^{-1} \cdot l^{-1}$. Despite the relatively high production, maximal efficiency of solar energy to H_2 conversion was only 0.042 %. Similarily, Masukawa et al [2002] demonstrated that the disruption of the uptake hydrogenase gene, but not the bidirectional hydrogenase gene, lead to enhanced photobiological hydrogen production by nitrogen-fixing cell cultures of *Anabaena* PCC 7120.

The importance of the bidirectional hydrogenase in cyanobacterial fermentation was recently demonstrated. The unicellular non-nitrogen-fixing cyanobacterium *Gloeocapsa alpicola* grown in the presence of limiting concentrations of nitrate is capable of intensive H_2 production during incubation under dark anoxic conditions [Troshona et al 2002]. This is the result of fermentation of glycogen accumulated at photoautotrophic growth. Besides H_2, acetate and CO_2 small amounts of D-lactate and ethanol are found as the products of fermentation. The fermentation stoicheometry, balances and enzymes activities indicate that the main pathway of glycogen degradation in *G. alpicola* is carried out via energy-yielding acetate formation involving pyruvate:ferredoxin oxidoreductase and acetate kinase. The removal of H_2 evolved from the culture, fermenting at elevated temperature and optimal pH with significant rate of argon flow allows H_2 production during at least ten hours at a significant rate.

FUTURE R&D AND INTERNATIONAL COOPERATIONS/NETWORKS

Biohydrogen is a long-term approach to develop knowledge and technologies aiming at producing molecular hydrogen from solar energy and water using a renewable process. The present R&D activities within this field, with its long-term goal, can be promoted, stimulated and made to advance at a faster rate through collaborations and networks. Besides some national biohydrogen programs and several individual projects, two major international initiatives can be recognized: the international program within IEA and the European program within COST.

In the International program IEA (International Energy Agency) Agreement of the Production and Utilization of Hydrogen, Annex 15 *Photobiological Hydrogen Production* the main objectives are to investigate and to develop processes and equipment for photobiological production of hydrogen by direct conversion of solar energy [Lindblad et al 2000].

In the European program COST (European CO-operation in the Field of Scientific and Technical Research) 8.41 *Biological and Biochemical Diversity of Hydrogen Metabolism* the main objective is to pool interrelated European expertise in order to understand the structural and molecular basis of the functions, as well as the factors that influence the activity and stability of hydrogenase enzymes.

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PHOTOBIOLOGICAL HYDROGEN PRODUCTION BY CYANOBACTERIA UTILIZING NITROGENASE SYSTEMS -PRESENT STATUS AND FUTURE DEVELOPMENT-

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ABSTRACT

In order to mitigate ever-increasing greenhouse gas concentrations in the atmosphere, development of renewable energy source sufficient in quantity is urgently required. The merits of photobiological hydrogen production by cyanobacteria utilizing their nitrogenase systems and the present status of the developmental research in our laboratory are discussed. Genetically defined hydrogenase mutants have been created, and disruption of uptake hydrogenase gene (Δ*hupL* mutant) has proven to be effective in elevating hydrogen productivity greatly in *Anabaena* sp. PCC 7120 cells. The efficiency of visible light energy conversion to H_2 by the Δ*hupL* mutant at its highest H_2 production stage was 1.0-1.6 % at incident light intensity of 50 W/m^2. At present, these activity levels do not last long, and energy conversion efficiencies decrease with further increase in incident light intensity. Several possible means of overcoming these difficulties for future development are discussed.

INTRODUCTION

Our needs for exploitation of renewable energies

Long records of atmospheric composition indicate an abrupt rise of atmospheric CO_2 concentration in the last 200 years. The average ground temperature of the Earth has increased to about 0.5°C during the 20th century, and this was the warmest of the past ten centuries (Fig. 1). This temperature rise is strongly correlated with anthropogenic activities of burning fossil fuels and of changing land uses. The IPCC report [1] concludes that there is a new and stronger evidence that most of the warming observed over the last 50 years is attributable to human activities. In order to stabilize greenhouse gas concentrations in the atmosphere at a level that would prevent dangerous anthropogenic interference with the

climate system (Fig. 2), development of renewable non-polluting energies alternative to or substituting for fossil fuels and with a considerable scale in quantity are urgently required.

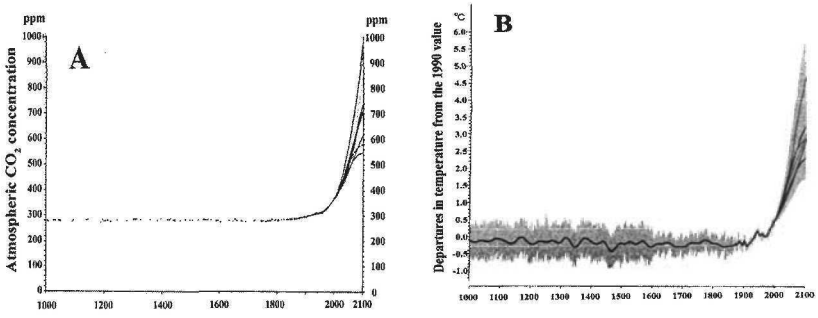

Figure 1. Observation of atmospheric CO_2 concentrations and Earth's surface temperature during the past 10 centuries and projections of them in the 21st century.
A: atmospheric CO_2 concentrations,
B: Earth's surface temperature Future projections differ depending upon emission scenarios. (adapted from IPCC Report 2001 [1])

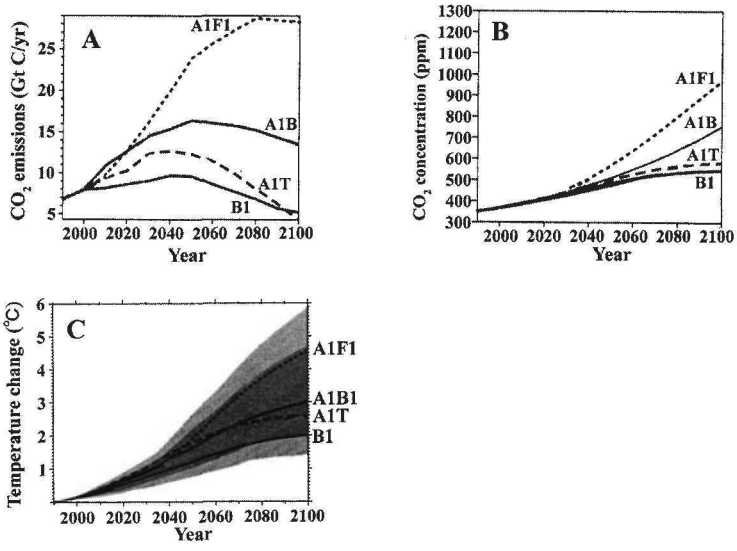

Figure 2. Projection of the global climate of the 21st century.
A: CO_2 emissions, B: atmospheric CO_2 concentrations,
C: Earth's surface temperature (adapted from IPCC Report 2001 [1]).
The global climate will depend on natural changes and the response of climate system to human activities. Four human activity models are indicated: A1F1, A1B, A1T, B1.

PHOTOBIOLOGICAL HYDROGEN PRODUCTION UTRILIZING CYANOBACTERIA

Merits of photobiological hydrogen production utilizing cyanobacteria

Solar energy is the most abundant renewable alternative, and hydrogen is the most likely environmentally friendly future fuel. The amount of solar energy received on Earth's surface amounts to more than 6,000 times of the world social energy consumption (Tab. 1). However, solar energy is diffuse in intensity and its economical utilization is not easy (Tab. 2). In order that H_2 substitutes for or supplements fossil fuels, its economical production is essential. Economical photobiological H_2 production is indeed challenging tasks, nevertheless it should be pointed out that efficient biological solar energy conversion is already realized to a certain extent in modern agriculture as exemplified by sugar production in sugarcane field at annual solar energy conversion efficiencies of about 1 %. The price of crude sugar in New York market has been in the range of 4.3-15.9 cents/lb in these ten years. A sugar price of 5 and 15 cents/lb corresponds to 1.5 and 0.5 MJ/cent or 2.4 and 7.2 cents/kWh in electric energy, respectively.

These figures indicate that commercially viable photobiological H_2 production processes may be attainable if we strive for it based on rigorous research and development strategies. For photobiological H_2 production, cyanobacteria are among the ideal candidates since they grow with minimal nutritional requirements (only mineral salts and water), with the sun light as the sole source of energy and with H_2 and O_2 as the major products [2, 3] (Fig. 3). In addition, the amount of the resource (substrate water) is almost unlimited. Furthermore, they pollute environment minimally because their waste bodies can be used to feed fishes [4].

Table 1. Quantity of Solar Energy is Huge

2.5×10^{24} J/year

		Quantity (10^{18} J/year)	Ratio (solar/social)
World			
	Social energy consumption (1996)	340	
	Photosynthesis (net production)	3,300	
	Solar energy	2,500,000	7,350
Japan			
	Social energy consumption (1996)	21	
	Solar energy (land)	2,100	100
	Solar energy (including EEZ*)	33,000	1,500

EEZ* : exclusive economic zone

Table 2. Economical Solar Energy Utilization is Challenging Tasks

Solar energy 1500 kWh/m^2/year (or 4 kWh/m^2/day)

Efficiency of energy conversion	Power rate per kWh	
	10 cents	40 cents
	earning/m^2/year	
1%	150 cents	600 cents
5%	750 cents	3,000 cents
10%	1,500 cents	6,000 cents

cf. sugar cane (about 1 % conversion efficiency)

sugar price: 4.3-15.9 cents/kg (NY market) or 2.0-7.6 cents/kWh

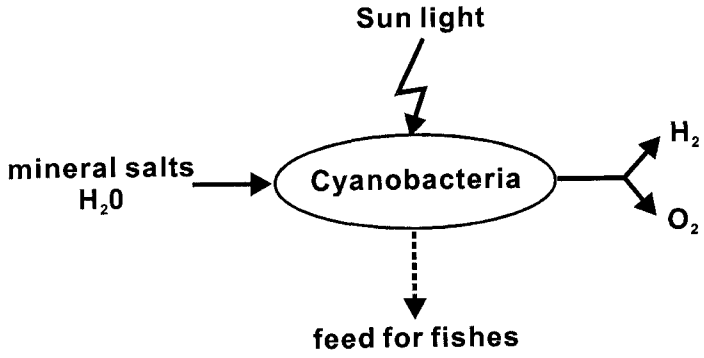

Figure 3. Photobiological H_2 production utilizing cyanobacteria. Cyanobacteria are ideal solar energy converters because they require only the sun, mineral nutrients, and water for H_2 production. Their waste bodies can feed fishes [4].

Enzymes of cyanobacteria involved in hydrogen metabolism

Cyanobacteria may possess several enzymes directly involved in H_2 metabolism: nitrogenase(s) catalyzing the production of H_2 concomitantly with the reduction of nitrogen to ammonia, an uptake hydrogenase, catalyzing the consumption of H_2 produced by the nitrogenase, and a bidirectional hydrogenase, which has the capacity to both take up and produce H_2 [3, 5].

The nitrogenase reaction is usually expressed as

$$N_2 + 8e^- + 8\,H^+ + 16\,ATP \rightarrow H_2 + 2\,NH_3 + 16\,(ADP + Pi) \qquad \text{Equation (1)}$$

in which hydrogen is produced as an inevitable byproduct [6].

Choice of nitrogenase-based hydrogen production

In cyanobacteria, hydrogen production is catalyzed either by nitrogenase or hydrogenase as stated above. H_2 production catalyzed by hydrogenase is superior in theoretical maximal energy conversion efficiency, but the need for periodic anaerobic conditions [7] makes this process less attractive. Although the nitrogenase reaction is a highly energy-consuming process, heterocystous N_2-fixing cyanobacteria have the unique ability to sustain H_2 production with the simplest of nutritional requirements. The merits of this system are that H_2 production is simultaneous with photosynthetic O_2 evolution, and no anaerobic treatment of the cells is required. We have chosen nitrogenase-based hydrogen production from a long-term point of view aiming at extensive farming of cyanobacteria.

Construction of hydrogenase mutants and effects of deletion of hydrogenases on hydrogen Production

One of the major obstacles to efficient photobiological production of H_2 in heterocystous cyanobacteria might be the presence of hydrogenases that reabsorb the H_2 produced by nitrogenase in the heterocysts [8]. Many heterocystous cyanobacteria contain both uptake hydrogenase (*Hup*) and bidirectional (or reversible) hydrogenase (*Hox*), though a few have only Hup [5]. In heterocystous cyanobacteria, Hup occurs predominantly in the heterocysts and is assumed to recover some of the H_2 produced by the nitrogenase reaction. *Hox* occurs in both vegetative cells and heterocysts, and is also considered to absorb H_2 due to its low K_m for H_2. As it is conceivable that hydrogenase could be beneficial to nitrogenase because it can protect O_2-sensitive nitrogenase by scavenging the O_2 diffusing into the heterocysts, the effects of the deletion of *hup* on nitrogenase activity need to be studied with genetically defined cyanobacterial mutants.

We have constructed three hydrogenase mutants from *Anabaena* sp. PCC 7120 (Δ*hupL,* Δ*hoxH* and Δ*hupL*/Δ*hoxH*), and evaluated the effects of deletion of hydrogenase genes on hydrogen production [9]. When cells were transferred to a combined nitrogen-free medium and grown under either air or air plus 1 % CO_2, Δ*hupL* and Δ*hupL*/Δ*hoxH* mutants attained the maximal H_2 producing activities significantly earlier than Δ*hoxH* and wild-type when cells were transferred to a combined nitrogen-free medium. The Δ*hupL* mutant produced H_2 at a rate 4-7 times that of wild-type under optimal conditions. The *hoxH* mutant showed significantly lower H_2 production and slightly lower nitrogenase activity compared with wild-type. The H_2 production activity of the Δ*hup*/Δ*hox* mutant was slightly lower than, but almost equal to, that of the Δ*hupL* mutant (Fig. 4).

Another merit of the Δ*hupL* mutant is that its activity of hydrogen reabsorption is very small even at high cell densities (Masukawa et al., unpublished observation). These results indicate that deletion of the *hupL* gene could be employed as a source for further improvement of H_2 production in a nitrogenase-based photobiological H_2 production system.

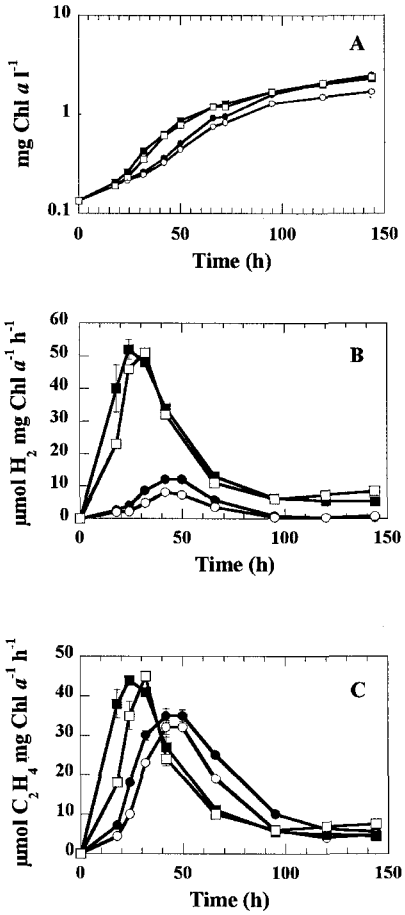

Figure 4. Growth curves of wild-type and mutants sparged with air. A Time course of in vivo B H_2-production activities and C nitrogenase of wild-type and mutants after transfer to $BG11_0$ medium. *Vertical bars* SD values for three replicates. ●Wild-type, ■Δ*hupL* mutant, ○Δ*hoxH* mutant, Δ*hupL*/Δ*hoxH* mutant (adopted from Ref. [9]).

Possible means of improving hydrogen production activity

For economical photobiological production of H_2 by cyanobacteria, we have tentatively set the following targets: energy conversion efficiency (vs. total solar radiation) of 1 %, and duration of such high H_2 productivity for several days.

The efficiency of light energy conversion to H_2 by the Δ*hupL* mutant at its highest H_2

production stage was 1.0-1.6 % at actinic visible light intensities (PAR) of lower than 50 W/m^2 under argon atmosphere, and the activity lasted for at least 35 min (Fig. 5). At 250 W/m^2, H_2 producing activity gradually decreased with illumination time [10]. Assuming that visible light energy (or photosynthetically active radiation, PAR) accounts for 50 % of total solar energy and that 10 % of radiation is lost by reflection at the surface, conversion efficiency (vs. PAR) of 1.3 % (mean of 1.0-1.6 %, see above) of Δ*hupL* mutant in the above described experiments corresponds to about 0.59 % of efficiency vs. total solar radiation. This value is not very far from our tentative target of 1 % but the problem is that the efficiency is lowered when light intensity (PAR) exceeded 50 W/m^2 [10]. Average total solar energy intensity received on the earth's surface in Japan is about 400 W/m^2 or about 200 W/m^2 PAR. These figures indicate the needs for further engineering of the low light-saturation properties of this strain or for search for cyanobacterial strains for better performance at high light intensities.

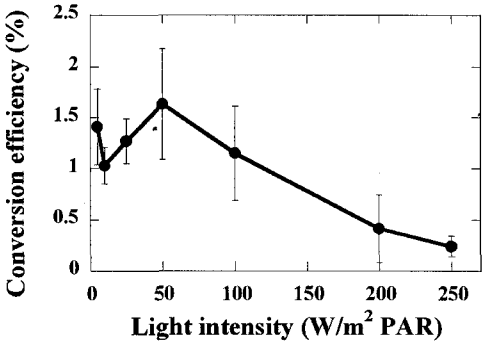

Figure 5. Effects of light intensity on energy conversion efficiency of photobiological H_2 production by Δ*hupL* mutant. Conversion efficiencies are calculated against absorbed visible light (PAR) energy with Δ*hupL* mutant at its nearly maximal. H_2 production stage. H_2 productions were determined between 10 and 35 min after onset of illumination. A: light intensity curve of H_2 production, B: Energy conversion efficiency [10].

H_2 production by Δ*hupL* mutant started to decline at about 30-40 hr after the Δ*hupL* culture was transferred to combined nitrogen-free medium (Fig. 1). In cyanobacterial cells used in our experiments, hydrogen is produced as a by-product of nitrogen fixation. When combined nitrogen is limited, cells vigorously fix nitrogen with simultaneous production of hydrogen. However, when nitrogen demand is satisfied and cell growth rates decreases, the rates of nitrogen fixation and of hydrogen production will decrease.

In order to achieve significant H_2 production rates over long time, cyanobacteria should be improved so that nitrogenase acitivty last longer. It should be pointed out that H_2/e^- ratio (or NH_3/e ratio) as exemplified in Equation (1) is not fixed. The equation represents a typical (or idealized for N_2 fixation) case. The NH_3/e^- ratio is called as "electron allocation coefficient" (typically 0.75). This value is reported to be modifiable downward (or upward for hydrogen production) under several conditions. Nitrogenase usually has Mo as the key element of the metal cofactor (Mo type nitrogenase). Some cyanobacteria have a V type

nitrogenase gene cluster, which is expressed under combined nitrogen deficient conditions when Mo supply is limited [11]. The electron allocation coefficient of the V type nitrogenase is in favor of hydrogen production (or in disfavor of nitrogen fixation) compared with that of the Mo type (conventional) nitrogenase. Some cyanobacteria may have the third nitrogenase activity (Fe-only type), which is expressed when supplies of both Mo and V are limited. The electron allocation factor of the Fe-only type nitrogenase, too, is assumed to be in favor of hydrogen production compared with that of the Mo type.

Modification of the electron allocation factor in favor of hydrogen production has dual merits: it will enhance hydrogen productivity and increase longevity of high nitrogenase level, because supply of combined nitrogen will be more difficult to be satisfied.

The electron allocation coefficient can also be modified by removing nitrogen gas from the gas phase after cells have developed enough nitrogenase activity, but we are not adopting this strategy because it will be against economical production of hydrogen from a long-term point of view.

For full activity of nitrogenase enzymes, they require homocitrate as a ligand for the Fe-Mo cluster. It has been shown with nitrogen fixing heterotrophic bacteria as well with in vivo experiments that the absence of homocitrate drastically decreases or abolishes nitrogen fixation with less effects on hydrogen production. Tricarboxylic acids (citrate etc.) can partially substitute for homocitrate. Genetic engineering to decrease in vivo homocitrate levels will be one of the means for enhancing hydrogen producing activity.

Site-directed mutagenesis of nitrogenase may be an additional possibility.

It is needless to say that the above developmental studies must be accompanied by intensive efforts of collecting many strains from various habitats and selecting potentially prospective strains among them.

CONCLUDING REMARKS

Using *Anabaena* sp. PCC 7120 as a model organism, for which whole genome has been recently sequenced [13], we have shown that disruption of the *hupL* gene leads to increase in H_2 production 4-7 times that of wild-type. The effectiveness of the above method should be investigated with various nitrogen-fixing cyanobacterial strains.

Using hydrogenase-disrupted mutants, further genetic engineering for improvement and longevity of hydrogen production seem to be promising. We will tentatively aim at energy conversion efficiency of about 1 % (vs. total solar energy) lasting for several weeks.

Apart from biological research, development of technology and accumulation of social capital are also required for the age of hydrogen energy. International cooperation is also a key factor. These concerted efforts will open the door for the age of renewable hydrogen energy (Fig. 6).

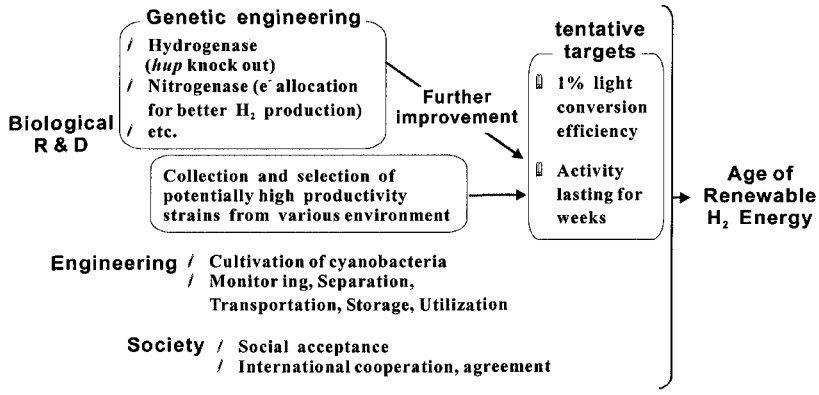

Figure 6. Schemes for development of photobiological H_2 as renewable energy.

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FUNDAMENTALS AND LIMITING PROCESSES OF BIOLOGICAL HYDROGEN PRODUCTION

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ABSTRACT

Biological hydrogen production has been known for over a century and research directed at applying this process to a practical means of hydrogen fuel production has been carried out for over a quarter century. The various approaches that have been proposed and investigated are reviewed and critical limiting factors for each are identified. The low energy content of solar irradiation dictates that photosynthetic processes operate at high conversion efficiencies and places severe restrictions on photobioreactor economics. Conversion efficiencies for direct biophotolysis are below 1 % and indirect biophotolysis remains to be demonstrated. Dark fermentation of biomass or wastes presents an alternative route to biological hydrogen production that has been little studied. In this case the critical factor is the amount of hydrogen that can be produced per mole of substrate. Known pathways and experimental evidence indicates that at most 2 to 3 moles of hydrogen can be obtained from substrates such as glucose. Process economics require that means be sought to increase these yields.

INTRODUCTION

Hydrogen production by biological systems has long been known and studied. Anecdotal reports of biological hydrogen production are over one hundred years old. Basic research on hydrogen production by bacteria started as early as the late 1920s [1] and hydrogen production by microalgae has been studied since the 1940s [2]. Research on applied aspects of biological hydrogen production began in the early 1970's and, combined with papers on more basic aspects, has resulted in literally hundreds of publications. Photosynthetic processes have been particularly emphasized, with dark fermentations being relatively neglected. However, over the past quarter century advances towards practical applications have been minimal. Here various types of proposed biohydrogen processes, including dark fermentations, are reviewed discuss some of the potentially limiting factors are discussed.

HYDROGEN EVOLVING ENZYMES

Of course the basis for any biological hydrogen producing system is an enzyme that is capable of carrying out what is arguably the simplest chemical reaction, the reduction of protons to hydrogen: $2H^+ + 2e^- \leftrightarrow H_2$. All enzymes capable of hydrogen evolution contain complex metallo-clusters as active sites and are synthesized in a complex processes involving auxiliary enzymes and protein maturation steps. At present three enzymes carrying out this reaction are known; nitrogenase, Fe-hydrogenase, and NiFe hydrogenase.

Nitrogenase is a two component protein system that uses MgATP (2ATP/e^-) and low potential electrons derived from reduced ferredoxin or flavodoxin to reduce a variety of substrates. In the absence of other substrates, nitrogenase continues to turnover, reducing protons to hydrogen. This is the basis for hydrogen production by nitrogenase-based systems. At each cycle, MgATP complexed Fe-protein associates with MoFe-protein, 2ATP are hydrolyzed with the transfer of one electron to MoFe-protein and the complex dissociates. Turnover is extremely slow, $6.4 \cdot s^{-1}$, necessitating the biosynthesis of enormous quantities of the two proteins. Biosynthesis of the complex metal centers requires a great deal of additional enzymatic machinery, energy, and time. Thus, considering the low turnover number, the considerable energy inputs necessary for biosynthesis and the requirement for ATP for catalysis, nitrogenase is not a very metabolically effective way to produce H_2.

Many microorganisms have been shown to contain a NiFe or NiFeSe hydrogenase which is usually thought of as functioning as an "uptake" hydrogenase whose normal metabolic function is to derive electrons from H_2 which are ultimately used to reduce NAD(P). The active site is a complex nickel iron center with coordination to 2 CN and one CO [3] and formation of active enzyme requires a number of additional enzymes catalyzing cluster biosynthesis and protein maturation reactions. Activities in the "uptake" direction are usually in the order of 300-400 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and rates of H_2 evolution by the purified enzyme are lower, 65 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ [4], which corresponds to a turnover rate of $98 \cdot s^{-1}$. Thus, even working in reverse this hydrogenase appears to be a better catalyst for hydrogen evolution than nitrogenase.

Fe-hydrogenase, which usually functions in the direction of hydrogen evolution, has been known for over 30 years. This enzyme contains a highly reactive complex Fe-S center in which one of the Fe atoms is complexed with CO and CN [5]. The Fe hydrogenases have extremely high turnover numbers: $6{,}000 \cdot s^{-1}$ for *C. pasteurianum* and $9{,}000 \cdot s^{-1}$ for *Desulfovibrio* spp. Note that this is a thousand times faster than the turnover number of nitrogenase!

Therefore the catalytic activity of these enzymes varies enormously and it is in theory possible that at least in some cases the quantity or activity of the hydrogen evolving enzyme could limit the overall process. However, there is little evidence for this being the limiting factor in any system. Indeed, in many microbial systems, potential catalytic activity far surpasses the amount of hydrogen produced, suggesting that other metabolic factors are limiting.

LIGHT-DEPENDANT PROCESSES

General Considerations

The low energy density of solar energy places severe economic restrictions on potential light-dependant biohydrogen processes. For example, at very favorable locations the yearly average solar irradiation could be as high as 5 $kWh/m^2/day$ which would give 6.6GJ/year.

At a very optimistic conversion efficiency of 10 % and a price for H_2 of \$15 per GJ only \$10 worth of H_2/m^2/year would be obtained thus severely constraining construction and operational expenditures. Obviously, the situation worsens if conversion efficiencies are lower. In spite of the critical importance of this consideration, relatively few studies on photobiological hydrogen production report conversion efficiencies, and when these are reported they typically fall well below 1 % (i.e. < \$1 H_2/m^2/year).

What are the factors that limit efficiencies and how could conversion efficiencies be increased? The general consensus is that in theory photosynthetic efficiencies could be as high as 10 % (conversion of total light energy into product). However, in practice nothing near this efficiency is obtained due to the rather low efficiencies typically observed at full sunlight intensities. This is because under full sunlight the overall rate of photosynthesis is limited by the rate of the dark reactions, which can be up to ten-times slower than the rate of light capture. Thus under full sunlight conditions up to 90 % of the photons captured by the photosynthetic apparatus are not used in productive photosynthesis but rather decay as heat or fluorescence. This light-saturation effect is therefore a major reason that photosynthetic productivities are not nearly as high as those projected from extrapolations of laboratory data at low light intensities.

One approach to overcoming this obstacle is to use light attenuation devices that transfer sunlight into the depths of a dense culture, for example arranging photobioreactors in vertical arrays to reduce direct sunlight, which greatly increases the requirement for costly photobioreactors, or using optical fiber photobioreactors, in which light energy is collected by large concentrating mirrors and delivered via optical fibers into small photobioreactors. Clearly both alternatives are technically and economically impractical.

Another approach is create mutant cells with reduced antenna pigment content. Cells with such a photosynthetic apparatus would theoretically absorb fewer photons at high light intensities, thus wasting fewer photons and becoming more efficient on a reaction center basis. This concept is supported by several lines of direct experimental evidence. Microalgal mutants with reduced antenna sizes have been produced and shown to have a 50 % increase in productivity in continuous laboratory cultures operating at high light intensities, compared to the wild type [6-8]. Similarly, other studies have used a physiological approach with cultures of the green alga *Dunaliella salina.* Under stress conditions this alga exhibited both damaged PSII centers and reduced antenna sizes, but when the stress was relieved PSII centers were repaired before their antenna sizes increased. This allowed for a brief period of relatively high rates of photosynthesis under high light intensities [9, 10]. On a more practical level, it is doubtful if such mutants could be maintained in any large-scale relatively open system since they would probably be out-competed by wild-type strains [11]. This is because in algal mass cultures cells are intermittently exposed to both high (at or near the surface) and, more frequently, low (deeper in the culture) light intensities, resulting in selection for strains with more light harvesting pigments per photosynthetic reaction center since overall these cells can capture more photons per cell than strains with a reduced ratio of antenna chlorophyll per reaction center.

Therefore the ultimate goal of photosynthetic H_2 production should be to increase solar conversion efficiencies. Currently, photosynthetic efficiencies, based on microalgal biomass production in outdoor cultures, of about 3 % of solar energy converted into algal biomass can be achieved. Increasing this by a factor of three-fold may be possible. Achieving this would make microalgal cultures even more efficient than sugarcane cultivation in tropical countries, which at present represents the highest productivity of any practical photosynthetic process. This goal is essential to the realization of practical microalgal processes for H_2 production. There are several mechanisms by which photosynthesis could yield H_2 and these are examined next.

Direct Biophotolysis

In direct biophotolysis the photosynthetic apparatus captures light and the recovered energy is used to couple water splitting to the generation of a low potential reductant, which can be used to reduce a hydrogenase enzyme. This is an inherently attractive process since solar energy is used to convert a readily available substrate, water, to oxygen and hydrogen: $2H_2O \rightarrow 2H_2 + O_2$ and was actually first demonstrated with a cell free system [12]. However, due to the inherent lability of the various components in a cell-free oxic environment, more reliable and robust systems using whole cells have been sought and studied since then by many researchers. Some green algae naturally possess an inducible reversible hydrogenase and in principle could be used in a direct biophotolysis scheme. However, this hydrogenase activity is extremely oxygen sensitive and attempts to achieve oxygen tolerant hydrogenase activity through classical mutagenesis have met with limited success [13]. Simultaneous hydrogen and oxygen production with this system has been demonstrated under very high inert gas sparging leading to very low concentrations of hydrogen (and oxygen) [14]. Although it has not been specifically studied, it appears that in order for there to be simultaneous H_2 and O_2 production the O_2 partial pressures must be below 0.1 %, which is less than one micromolar O_2 in the liquid phase. It would not be possible to maintain such low partial pressures by high rate sparging in any practical direct biophotolysis process, due to the large amount of diluent gas and power inputs required for gas transfer.

Another approach to overcoming the limitations inherent in the use of the highly oxygen sensitive hydrogenase in a process in which oxygen is involved is to remove the oxygen through cellular respiration. This has been demonstrated with a hydrogen production process based on the green alga *Chlamydomonas reinhardtii* [15], although this process was originally purported to be a two-stage indirect process. As presently studied, this system relies on growth on acetate to increase respiratory activity and starvation for sulfur to decrease photosystem II activity (the water-splitting reaction) by over 80 %. The increased respiratory activity is sufficient to handle the consequently reduced rates of oxygen evolution thus maintaining dissolved oxygen levels low enough to permit sustained hydrogenase activity. It is clear that in this system most of the electrons used to drive hydrogen evolution are derived from the residual photosystem II activity [16]. Obviously this system suffers from several major drawbacks. For one thing, the use of acetate and the requirement for pumping between stages is probably impractical in practice. More importantly, reductant which could other wise be used in hydrogen production is "wasted" through respiration thus reducing overall efficiency. Finally, this system relies on a 4 to 5 fold reduction in photosynthetic capacity in order to reduce oxygen evolution to tolerable levels. Clearly, as discussed previously, in light of the need to increase photosynthetic efficiencies, this is an unacceptable tradeoff. Thus, the O_2 sensitivity of the hydrogenase enzyme reaction and supporting reductant generating pathway remains the key problem in green algal hydrogen production, as it has been for the past thirty years.

One approach that at first glance appears to provide a solution to the problem of oxygen sensitivity is the use of heterocystous cyanobacteria. In these organisms, composed of two distinct cell types, heterocysts and vegetative cells, the hydrogen evolving and oxygen evolving activities are spatially separated. In these organisms hydrogen evolution is catalyzed by nitrogenase, which is confined to the heterocyst, a dedicated cell type that has a number of specific features that provide oxygen protection for the nitrogenase enzyme. Hydrogen production by these organisms has been amply demonstrated under both laboratory and outdoor conditions [17-22]. However, several factors, both theoretical and practical, appear to limit hydrogen production, including limitations inherent in the nitrogenase system; oxygen sensitivity, low turnover number, a requirement for ATP, as well as the additional energy costs involved in heterocyst differentiation and maintenance. In addition, maximal

conversion efficiencies are obtained only at low solar flux [21], highlighting the inability of these organisms to completely utilize full solar intensity. Taken together, the overall maximum efficiency of this system is probably well below that which is required for a practical system.

An additional problem with any direct biophotolysis is that it requires large photobioreactors and the separation of H_2 and O_2, the costs of which might easily make such a process impractical. In direct biophotolysis the entire production area must be enclosed in photobioreactors able to both produce and capture H_2 and O_2. However, few reliable cost estimates are available for large-scale enclosed photobioreactors. Even inclined tubular photobioreactors with internal gas exchange [23], or other similar systems, impermeable to hydrogen, are likely to cost at least $\$100/m^2$, without gas separation or even contingencies and engineering costs [23]. Given the severe economic constraints imposed by solar flux and photosynthetic efficiencies, discussed above, it would appear that any single-stage process, such as direct biophotolysis or heterocystous cyanobacterial processes, would not be cost effective. Therefore, direct biophotolytic processes, though inherently attractive, appear to suffer from the insurmountable barriers of oxygen sensitivity, intrinsic limitations in light conversion efficiencies, problems with gas capture and separation, and very onerous economics.

Indirect Biophotolysis

In indirect biophotolysis problems of the sensitivity of the hydrogen evolving process are potentially circumvented by separating temporally and/or spatially oxygen evolution and hydrogen evolution. Thus indirect biophotolysis processes involve the separation of the H_2 and O_2 evolution reactions in different stages.

One process based on this concept for example [25] would involve; first, the production in open ponds of biomass high in stored carbohydrates (water splitting photosynthetic step). Then the biomass would be suitably concentrated, for example in settling ponds, and then subjected to an anaerobic dark fermentation to yield 4 H_2 /stored glucose plus 2 acetates. The cells could then be transferred to a photobioreactor in which the algal cells would use light energy to convert the two acetates to 8 moles of H_2. The economics of such a system are unknown and likely to be unworkable given the need for pumping between stages, the requirement for acheiving very high solar conversion efficiencies and high carbohydrate content in the first stage, and the relatively large photobioreactor area that is probably necessary for the second stage.

However, this, and other, indirect biophotolysis processes are still at the conceptual stage. As noted above, a hydrogen production process using sulfur deprived *Chlamydomonas reinhardtii* was originally purported to be an indirect biophotolysis system [15], but has subsequently been shown to be a direct biophotolysis process in which photosynthesis is greatly reduced in the second, anaerobically adapted stage. There is little evidence for appreciable carbohydrate storage in the first stage. In conclusion, indirect biophotolysis processes are of questionable economics and remain to be demonstrated even on an experimental level.

Photofermentations

Photosynthetic bacteria have long been studied for their capacity to produce hydrogen through the action of their nitrogenase system. These bacteria carry out anoxygenic photosynthesis with the photosynthetic apparatus supplying energy in the form of ATP and the electrons necessary for proton reduction ultimately coming from an organic substrate.

The photosynthetic bacteria have been shown to produce hydrogen from various organic acids and food processing and agricultural wastes. Calculations of the efficiency of conversion of light energy into hydrogen often show values approaching 100 %, but these estimates generally ignore the energy content of the organic substrate. True photosynthetic efficiencies are much lower under ideal (low) light conditions. Although little data is available, photosynthetic efficiencies must be even lower under high light (full sunlight) conditions since the photosystem of the photosynthetic bacteria is, like that of microalgae and cyanobacteria, optimized for low light conditions. As previously discussed, the photosynthetic efficiencies of this system in theory could be improved by the appropriate genetic manipulation of antenna pigment content. Although reported hydrogen production yields are impressively high with essentially stoichiometric conversion of substrates to hydrogen, there are several central issues that negatively impact the potential use of photosynthetic bacteria.

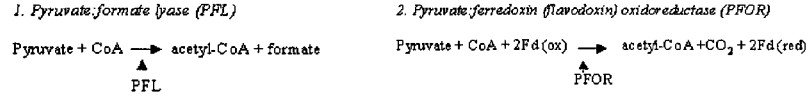
The critical issues are: 1) the low solar energy conversion efficiencies; 2) the use of the nitrogenase enzyme with its inherently high energy demand (as detailed above); and 3), as with any light driven system, the requirement for elaborate anaerobic photobioreactors covering large areas. Indeed, after extensive research and development research in several parts of the world, this process has been largely dropped from further study.

In conclusion, the rates and efficiencies of hydrogen production by systems directly using photosynthesis fall short of even plausible economic feasibility. Since organic substrates are the ultimate source of hydrogen in photofermentations or indirect biophotolysis processes, it can be argued that it should be simpler and more efficient to extract the hydrogen from substrates using a dark fermentation process, an approach which is discussed next.

DARK FERMENTATIONS

Fermentative processes can use either biomass obtained in a first stage light conversion process (e.g. higher plant or microalgae biomass high in starches or other fermentable substrates) or perhaps more attractively, various waste streams. These present an interesting yet largely unexplored avenue for the biological production of hydrogen. Much is presently known about the molecular biology and biochemistry of the hydrogen producing enzymes, reductant generating systems, and physiology of many hydrogen-producing fermentative microorganisms [26]. The enormous potential of metabolic engineering for redirecting electron flux to hydrogen production in various microorganisms remains to be exploited [27]. Very little is presently known about the currently attainable yields or the possibilities for improving these yields in the future. These questions are under active study.

The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates. The breakdown of pyruvate is catalyzed by one of two enzyme systems:



Thus in both these biological systems, the pyruvate generated by glycolysis is used, in the absence of oxygen, to produce acetyl CoA, from which ATP can be derived, and either formate or reduced ferredoxin (Fd (red)), from which hydrogen can be derived. The enteric bacteria derive hydrogen from formate and strict anaerobes derive hydrogen from Fd (red).

The overall yields in these metabolisms are relatively low; one to two hydrogen produced per molecule of pyruvate. For one thing, this is a natural consequence of the fact that fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate (pyruvate) is used in both cases to produce ATP giving a product (acetate) that is excreted. Also, in many organisms the actual yields of hydrogen are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume a portion of the hydrogen produced. It is unknown to what extent hydrogen production could be increased through metabolic engineering and manipulation of culture conditions.

One fermentation of interest would be to harness the endogenous metabolic machinery of algal cells to quantitatively convert stored carbohydrate (starch or glycogen in green algae and cyanobacteria, respectively) to H_2 in a strictly dark reaction. This would therefore be similar to the indirect biophotolysis concept outlined previously, but would effectively eliminate the photobiological stage, that is the photobioreactors, the major limitation in any practical biophotolysis process. In effect, the overall process would be simplified to that of algal biomass production in large open ponds, under conditions inducing high content of carbohydrates, followed by a dark fermentative stage.

The major issue is the feasibility of a dark fermentative reaction yielding close to the 12 moles of H_2 stored in each molecule of glucose metabolized. From a thermodynamic perspective, the most favorable products from the breakdown of one mole of glucose are two moles of acetates and four moles of H_2. However even for four H_2 a near-stoichiometric yield is only achievable at very low partial pressures of H_2. The challenge in this field is therefore to find ways to readily achieve four H_2 per glucose or possibly to exceed this amount and achieve nearly complete conversion of substrate to hydrogen. Even conversion of substrate to four H_2 would represent a major advance and would possibly allow the development of a practical system of two stage fermentation with conversion of substrate, including various waste streams, to hydrogen and acetate in the first stage. Then, in a second stage, the acetate could be converted to methane. Such a two stage system would therefore provide separate gas streams of hydrogen and methane which could be used in separate applications or combined to form a hythane mixture which has certain advantages as a carburant. Further research is needed to ascertain if such yields are indeed attainable in practical fermentations of appropriate feed streams.

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IV. Bio Molecular Device

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THE ISOLATION OF GREEN ALGAL STRAINS WITH OUTSTANDING H_2-PRODUCTIVITY

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ABSTRACT

Chlorophycean [Fe]-hydrogenases are simple structured enzymes that catalyze H_2-evolution with similar rates to the more complex [Fe]-hydrogenases from bacteria. Physiological studies indicate that these [Fe]-hydrogenases are quite common among green algae. After the identification of the *hydA* genes from *Chlamydomonas reinhardtii* ("Reinhardtii"-clade) and two scenedesmacean species ("Scenedesmus"-clade) we now introduce a third subtype, isolated from *Chlamydomonas moewusii* which represents the "Moewusii"-clade of green algal phylogeny. As in the cases of *C. reinhardtii* and *S. obliquus*, two different variants of the *hydA* gene could be identified in *C. moewusii*, and at least one *hydA* gene contributes to the extraordinary high H_2-production activity of anaerobically induced *C. moewusii* cultures. Another possibility to optimize the H_2-productivity of green algae lies in the creation and identification of mutants that affect the physiological hydrogenase activity. We therefore tested a new mutant screening system which enables to accomplish a quantitative hydrogenase activity assay in green algae.

INTRODUCTION

In times of scarce fossil resources and recurring discussions about the danger of radioactive waste and the effects of environmental stress and exploitation, it becomes obvious that future energy sources have to be unlimited, renewable and environmentally neutral.
H_2-photoproduction by green algae from water and sunlight perfectly meets all the above mentioned demands but the limited productivity of a given culture and the usually high oxygen sensitivity of the H_2-producing [Fe] - hydrogenase [Ghirardi et al., 2000; Melis and Happe, 2001] are still problems for efficient biotechnological applications.

[Fe] - hydrogenases from green algae are small monomeric enzymes of 44-55 kDa which catalyze H_2-evolution with similar efficiency as the more complex [Fe]-hydrogenases from prokaryotes [Peters et al., 1998]. Recent physiological examinations indicate that this type of enzyme is quite common among green algal species. Although some algae seem to lack the typical [Fe]-hydrogenase phenotype [Winkler et al., 2002a], a lot of species have been described to develop a more or less efficient H_2-production activity under anaerobic or sulfur

deprived conditions [Happe et al., 2002]. The first chlorophycean *hydA* gene was identified in *Chlamydomonas reinhardtii* [Happe and Kaminski, 2002] being a representative of the "Reinhardtii"-clade of chlorophycean phylogeny [Proeschold et al., 2001].

Another *hydA* subtype was found in *Scenedesmus obliquus* [Florin et al., 2001] and *Chlorella fusca* [Winkler et al., 2002a] which both belong to the "Scenedesmus"-clade. Under anaerobic conditions neither *S. obliquus* nor *C. fusca* reach the H_2-production efficiency of *C. reinhardtii.*

Furthermore the hydrogenase activity of *S. obliquus* is scarcely induced by S-deprivation [Winkler et al., 2002b]. Up to now *C. reinhardtii* seems to be the most promising species for biotechnological H_2-production.

Here we introduce a third algal *hydA* subtype, isolated from *Chlamydomonas moewusii* which represents the "Moewusii"-clade of green algal phylogeny [Proeschold et al., 2001]. Physiological measurements with additional chlorophycean species revealed, that *C. moewusii* rapidly develops an extraordinary high *in vitro* H_2-production activity under anaerobic conditions. Two different types of the *hydA* gene could be identified in the genome of *C. moewusii.*

Beside the screening for additional H_2-producing algal species, interests have been focussed on the identification of mutants that possess an aberrant hydrogenase phenotype. Such abnormities in H_2-metabolism help to understand the induction process of hydrogenase activity in favour to optimize the physiological hydrogenase productivity. One possibility is to generate mutants and perform a screening for an unusual hydrogenase activity with the intention to identify genes that influence the enzymatic activity of the [Fe]-hydrogenase. Various approaches have been undertaken to create a screening system with a reliable hydrogenase activity assay, i.e. by using a chemochromic film [Flynn et al., 1999] or H_2 reduced methylen blue [Fodor et al., 2001] as a reporter element. Here we present a fast and facile method, which not only allows the detection of a higher or lower H_2-production level but also offers a possibility to quantify the respective hydrogenase activity. A similar screening system was also established with cyanobacteria [Schmitz, Boison and Happe, unpublished].

MATERIALS AND METHODS

Algae strains and growth condition

Chlamydomnas moewusii strain SAG 24.91 was obtained from the working group of Prof. M. Melkonian [Botanical Institute, University of Cologne]. The cultivation of *C. moewusii* was done photoheterotrophically in liquid TAP (Tris acetate phosphate) medium [Harris, 1989] and on TAP medium plates with 1 % of agar. *Chlamydomonas reinhardtii* strain *CW⁻388⁻ (cw15 arg7 nitI mt⁺)* was grown in TAP medium supplemented with 50$\mu g \cdot ml^{-1}$ arginine and 1 % sorbitol [de Hostos et al., 1989].

The cultures grew at 18-20°C under a constant illumination (150 μmol photons$\cdot m^{-2} \cdot s^{-1}$) provided by cool-white fluorescence lamps. For the anaerobic induction, cells were harvested by centrifugation (5 min, 3000×*g*) in the exponential growth phase at an approximate culture concentration of 2×10^6 cells$\cdot ml^{-1}$ and afterwards resuspended in fresh TAP medium.

Cell Count and Chlorophyll Determination

Pigments from cells or thylakoid membranes were extracted in 80 % acetone and debris was removed by centrifugation at 10,000×*g* for 5 min. The absorbance of the supernatant at 652 nm was measured by a Shimadzu UV-visible spectrophotometer. The chlorophyll (*a* and *b*) concentration of the samples was determined according to Arnon [1949], with equations corrected as in Melis et al. [2000].

Isolation of the hydA gene by PCR methods

For the specific amplification of *C. moewusii* genomic *hydA* DNA, a *C. moewusii* colony was scraped from an agar plate and resuspended in 10 mM Tris-HCl, 10 mM EDTA and 150 mM NaCl. After disrupting the cells (Cell disruptor B 15, Branson, Danbury, USA), 2 µl of the resulting suspension was used directly in the PCR.

Four oligonucleotides were derived from the alignment of 3 chlorophycean *hydA* cDNA sequences (*hydA1* from *C. reinhardtii*, *hydA1* from *S. obliquus*, *hydA* from *C. fusca*). The sequences of the degenerated forward primers (primer 1 and primer 3) were: GCGTGTCGCTATTGCAGAGACC and ASGAGCCRCTGCCCATGTTCACCA For the amplification of the *hydA1* fragment primer 2 (GTGCGMAGYGCYGCCTCCAT) was used in both cases as the corresponding reverse primer while for the isolation of the *hydA2* fragment primer 2 was used for the first and primer 4 (CCKCCRGTGGTGCCRAACAG) for the nested PCR. Primer 1 and primer 2 were used for the first PCR, while the primers 3 and 2(4) were chosen for the nested PCR using 1µl of a 1:10 dilution from the first PCR. The PCR conditions were the same as described before [Winkler et al., 2002a].

RACE-PCR was performed with the SMART^{TM}RACE cDNA Amplification Kit (Clontech, Palo Alto, USA) according to the producers manual. 1µg mRNA derived from an anaerobically adapted *C. moewusii* culture was used as template for the generation of the cDNA first strand. For the 3'RACE procedure, sequence specific primers (GSPs) were derived from the corresponding genomic DNA fragment and later on used as sense primers together with the UPM or NUP, respectively. For the *hydA1*-3'RACE-PCR primer 5 (GCCGAAGAGGCTGGTGTGAA)was utilized as GSP1 and primer 6 (CTGGTGTGAAGCCTGGTGAC) as GSP2. Primer 7 (TGGCCACTACACAAGATGGAC) and primer 8 (TGTTTGCTGAGCGGCAGATT) were the GSP1 and GSP2 primers in the *hydA2*-3'RACE-PCR.

The sequences of genomic and cDNA fragments were determined using the dideoxynucleotide chain-termination method performing a cycle sequencing reaction with the BigDye-Sequencing Kit [Applied Biosystems, Weiterstadt, Germany]. The separation and detection of the labelled amplification products was done by a capillary sequencer (ABI Prism 310). Sequence analyses and homology tests were performed using the Sci Ed Central program package (Scientific Educational Software) and the Blast server [Altschul et al., 1985] of the National Center for Biotechnology Information (Bethseda, MD) was used for database searches. The online alignment service Clustal W [Thompson et al., 1994] was used for the determination of evolutionary relationships between related polypeptides.

Isolation and analysis of RNA

Following a procedure modified from the protocol of Newman [Newman et al. 1990], total RNA was extracted from 100 ml *C. moewusii* culture after 2 hours of anaerobic adaptation. The mRNA was isolated from 250 µg of total RNA using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). 50 µg RNA isolated from anaerobically induced cultures of *C. moewusii* (1 h, 2 h, 3 h and 4 h) were separated on a 0.8 % agarose gel

containing 1 % (v/v) of formaldehyde and transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotechnology, Uppsala, Sweden). The RNA probe was generated and labelled with digoxygenin (DIG) by *in vitro* transcription using the DIG RNA labelling kit of Roche (Roche Diagnostics, Mannheim, Germany). The RNA blot was washed with 0.1×SSC and 0.1% SDS at 68°C.

Nuclear transformation of C. reinhardtii

Mutants were generated using the glass bead transformation method according to Kindle [Kindle et al.1990]. The samples were spread out on TAP-agar plates lacking arginine and afterwards incubated at 18°C under a constant cool light illumination of 50µmol photons·m^{-2}·s^{-1}. After 2 weeks the transformant colonies were picked for further analysis and transferred to fresh TAP agar plates. A library of about 1500 nuclear transformants was obtained and used for screening.

Hydrogen evolution assay in deep well plates

Standard 2 ml deep well microtiter plates (VWR, Darmstadt, Germany) were filled with 1.6 ml TAP agar. Mutants were picked from plates with inoculation loops and dispensed with glass beads on the agar surface of single wells. The sides of the plates were wrapped with aluminium foil to prevent a stronger illumination of the wells at the edge. The microtiter plates were incubated under the same growth conditions as described above until the cells formed a homogenous layer inside the wells. For induction of hydrogenase, the plates were exposed to anoxic conditions inside an anaerobic chamber for 1 h. Hydrogenase activity of *CW388*$^+$ was determined *in vitro* with reduced methyl viologen using a gas chromatograph (Hewlett Packard 5890 A Series II, column: molecular Sieve 5 A, Mesh 60/80). Each well was filled with 200 µl of a reaction mix (20 mM $Na_2S_2O_4$; 5 mM methylviologen; 10 % Triton X100 and 50 mM potassium phosphate buffer pH 6.8) under anoxic conditions using a multipipette.

The plates were sealed with a MicroMat^{TM} (SCP, Weiterstadt, Germany) and incubated for 30 min at 37°C. For the measurement of hydrogenase activity, 200 µl gas phase was drawn from each well and analysed by gas chromatography. Due to the fact that hydrogen evolution proceeds during the measurements, the value of a well was compared to the corresponding activity from a standard deep well plate with wild type cultures for evaluation. The exact quantification of the hydrogenase activity was possible by measuring the optical density of each well. To verify the preliminary results, the interesting strains were further examined in an extended anaerobic assay, using a 50 ml culture of the respective transformant which was bubbled with argon gas [Happe and Naber, 1993; Happe et al., 2002].

RESULTS

Isolation and characterization of the [Fe]-hydrogenase gene from C. moewusii

After the alga *C. moewusii* was anaerobically flushed with argon, the cells produced hydrogen gas very rapidly after 15minutes. This anaerobic adaptation experiments performed with various chlorophycean species demonstrated that cultures of *C. moewusii* develop an extraordinary high *in vitro* hydrogen production activity of about 400 nmol H_2·µg Chl^{-1}·h^{-1} which is 2-3 times higher than the maximal activity of *C. reinhardtii* (Fig. 1A).

To isolate the cDNA of the presumed [Fe]-hydrogenase gene, PCR was performed using

a single disrupted *C. moewusii* colony as template material. Doing primordial PCR with primers 1 and 2 and the nested PCR with the primer combination 3/2 resulting in a main product of 0.6 kb, which was sequenced. By comparing the presumable *hydA*-fragment (*hydA1*) with the corresponding cDNA region of other *hydA* genes it was possible to divide the genomic fragment in two exon and two intron regions. For cDNA isolation, the *hydA1* specific primers 5 and 6 were generated corresponding to sequences of the second exon. A second genomic *hydA* homologue fragment of 0.9 kb was isolated from a nested PCR with the primer combination 3 and 4. This second amplified region corresponds partially to the first fragment, but possesses characteristic differences, especially in the presumable intronic regions. Therefore it was concluded that the second fragment belongs to another *hydA* gene (*hydA2*). This genomic region presumably comprises three exons and three introns. The sequence of the second exon of fragment 2 enabled the generation of the *hydA2* specific primers 7 and 8.

To obtain the complete cDNA sequences of *hydA1* and *hydA2*, RACE-PCR was performed using 1 µg of mRNA isolated from an anaerobically adapted culture of *C. moewusii*. Using the GSP primers 5 and 6 one after the other for the 3'RACE-PCR it was possible to isolate a 1.2 kb fragment of the 3' end of the *hydA1* cDNA. Furthermore it was possible to obtain a 0.8 kb fragment of the 3'end of the *hydA2* cDNA, with primer 7 and 8 (nested PCR) as GSP1 and GSP2, respectively. Both fragments were sequenced, which in the case of *hydA1* revealed 764 bp of the *hydA1* open reading frame coding for 254 amino acids (aa) followed by a 0.4 kb 3'untranslated region (UTR) which is terminated by a polyA tail. The 3'RACE fragment of *hydA2* consists of a 614 bp ORF (204 aa) and a 161 bp 3'UTR.

Using 0.2 kb of the 3'UTR region of *hydA1*, a RNA probe was generated and hybridised with total RNA which had been derived from 50 ml culture samples after 0, 1, 2 and 4 hours of anaerobic induction. The Northern blot results demonstrate that *hydA1* is highly transcribed under conditions of anaerobic adaptation (Fig. 1B).

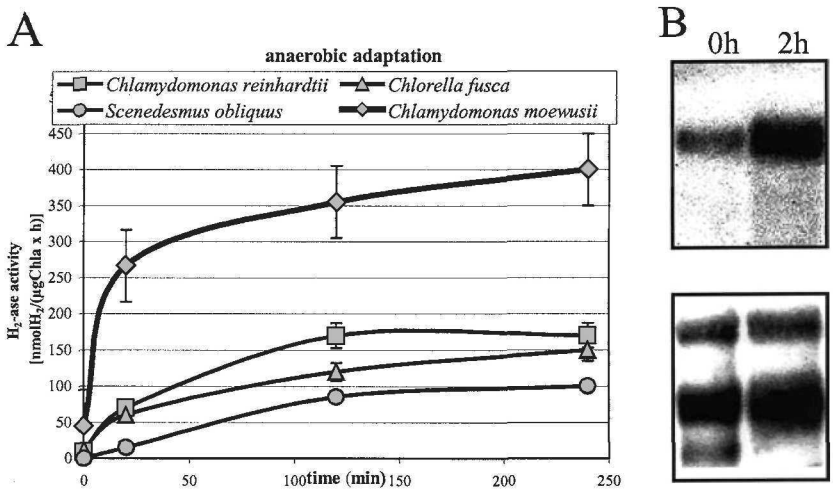

Figure 1. Hydrogenase activity upon anaerobic induction.
A: Anaerobic adaptation was obtained by flushing the cells with argon. At indicated time points, samples were taken to measure the *in vitro* H_2-ase activity of *C. reinhardtii* (box), *S. obliquus* (circle), *C. fusca* (triangle), *C. moewusii* (diamond). While the activities of both *Scenedesmus* species are comparative low, the *in vitro* H_2-production rate of anaerobically induced *C. moewusii* cultures is 2 times higher than the activity of induced *C. reinhardtii* cultures. B: Northern blots with equal amounts of total RNA isolated from an anaerobically adapted culture (2 h) and an uninduced reference culture (0 h) of *C. moewusii*. The upper blot was incubated with a RNA sample of the 3'UTR of the *hydA1* cDNA, while the lower blot was incubated with a RNA sample generated of the cDNA from the constitutively expressed sedoheptulose 1,7-bisphosphatase gene.

Structural characteristics of HydA from C. moewusii

By adding the non overlapping part of the 3'cDNA terminus to the presumable exonic sequence of the genomic *hydA1* fragment, a preliminary cDNA sequence of 1296 bp can be deduced coding for 297 amino acids. Following the same procedure one can further derive a preliminary 1022 bp cDNA fragment for *hydA2*, which codes for 287 amino acids. Amino acid alignments with different chlorophycean *HydA* variants based on 282 aa from the C-terminus of the respective *HydA* proteins revealed an identity of 83 % between both *C. moewusii HydA* sequences, while the sequence-identity with other algal *HydA* proteins lies between 66 % (*C. moewusii HydA*1; *S. obliquus HydA*1) and 47 % (*C. moewusii HydA*2; *C. reinhardtii HydA*1).

Within the confidently identified sequences of both proteins, all three of four cysteine residues which are involved in the structural integration of the H-cluster (active center consisting of one [4Fe4S] subcluster and a special [2Fe2S] subcluster) are present (Fig. 2).

Furthermore, all highly conserved amino acid positions, that are essential for the formation of the H-cluster containing hydrophobic niche or essentially involved in the proton transfer process can be found at their expected position in the sequences of both *HydA* fragments.

Comparisons with non-chlorophycean [Fe]-hydrogenases permitted the identification of an additional polypeptide region comprising of 15 aa in the middle part of both C-terminal *HydA* fragments which up to now has only been found in the [Fe]-hydrogenases of green algae. As this region normally consists of more than 20 aa (i.e. 54 aa in the case of *HydA*2 from *C. reinhardtii*) *C. moewusii* possesses so far the smallest insertion among the chlorophycean *HydA* proteins. Beside this difference regarding the length and also the composition of this insertion in comparison to the other chlorophycean *HydA* subtypes, there are some unusual deviations and additional or displaced cysteine residues within the minor conserved parts of *HydA*1 and *HydA*2 from *C. moewusii.* Preliminary protein models of *HydA*1 and *HydA*2 demonstrate some presumable 3 dimensional effects of one or the other sequence variation (data not shown).

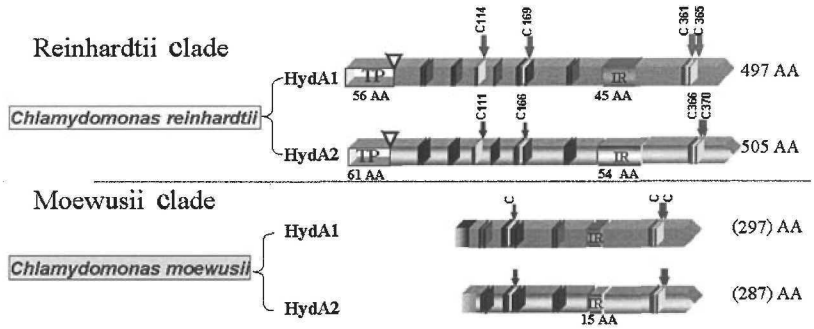

Figure 2. Schematic amino acid sequence comparison between the two [Fe]-hydrogenases from *C. reinhardtii* and the partial sequences of *HydA*1 and *HydA*2 from *C. moewusii.*

The *HydA*1 polypeptides are represented by dark gray bars,white the light gray bars depict the *HydA*2 variants. Cysteine residues which are involved in the integration of the [Fe-S]subclusters of the H-cluster are symbolized by bright squares. Dark gray squares indicate amino acid positions that essentially participate in the construction of the hydrophobic niche which contains the H-cluster or they mark residues which mediate the proton transfer from the molecular surface to the active center. The white triangles indicate the cleavage site of the signal peptidase which removes the transit peptide (TP). The insertional peptide region (IR) is indicated within each *HydA* (bar). The characterized parts of the *HydA*1 und *HydA*2 molecules from *C. moewusii* comprise nearly all highly conserved amino acid residues which are essential for establishing a functional [Fe]-hydrogenase.

Molecular phylogenetic analyses

The alignment file calculated with Clustal W was used to generate a phylogram, which depicts the relative evolutionary relationships between all characterized *HydA* sequences (Fig. 3). Corresponding to recent 18S SSU rRNA phylograms regarding the phylogeny of

Chlorophycean species [Proeschold et al., 2001], the *HydA* proteins of *C. moewusii*, *C. reinhardtii* and *S. obliquus* form 3 different branches which possess, one to the other, a rather similar evolutionary distance. The three different HydA branches reflect the membership to three distinct phylogenetic clades ("Moewusii"-clade, "Reinhardtii"-clade, "Scenedesmus"-clade) which can be found in completely different regions of the chlorophycean phylogenetic tree.

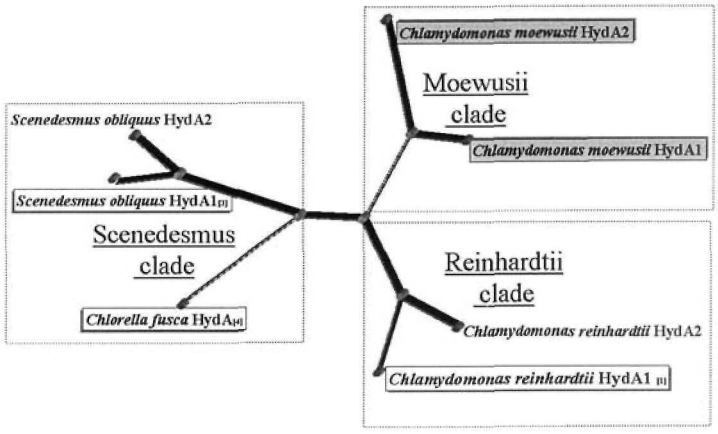

Figure 3. Evolutionary relationship between all characterised members of the chlorophycean [Fe]-hydrogenase family.
The phylogram is based on the alignment of 287 amino acids in the C-terminal part of the different *HydA* sequences. The sequences were aligned by CLUSTAL W [Thompson et al., 1994].
The unrooted tree was generated using the program Phylo Draw.
Names and origins of the different *HydA* proteins are given at the end of each branch. The names of protein sequences derived from genes which were identified in our lab are underlined with bright or dark gray boxes. The big blank boxes delimit specific clades of the chlorophycean phylogeny defined by Proeschold [Proeschold et al., 2001] according to a phylogenetic tree based on 18 S rRNA.

Generation of a mutant library and screening of the transformants

Using the transformation method established by Kindle [Kindle et al., 1990], a library of about 1500 *CW388* arg^+ mutants was created. After transformation with plasmid pARG 7.8 carrying the *arg7* wild-type gene of *C. reinhardtii* [Debuchy et al., 1989] mutants of *C. reinhardtii* lacking argininosuccinate lyase were complemented and grown on TAP medium in the absence of arginine (arginine prototrophs; Fig. 4).

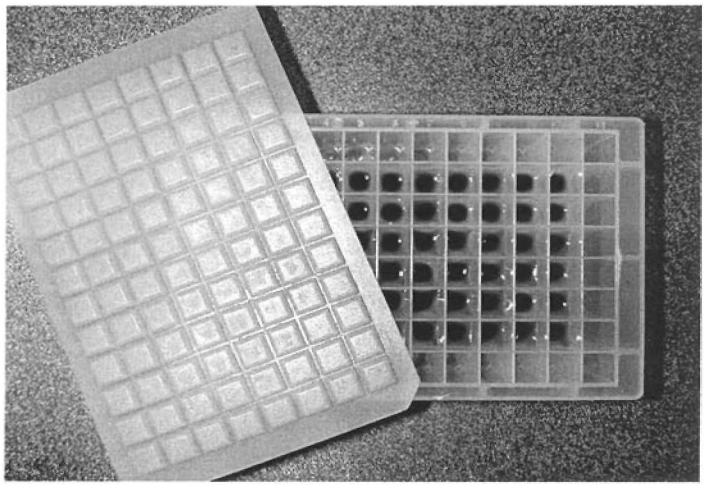

Figure 4. Photograph of a two ml deep well plate with MicroMap™ used for the mutant screening.
The photograph shows the two ml standard deep well plate with 1.6 ml TAP-agar. Transformant colonies where picked from plate, spread inside the wells with plating-beads and incubated for 12 days at 20°C under illumination. The plate was afterwards sealed with a MicroMat™

Due to anaerobic incubation, the [Fe]-hydrogenase was induced and its activity determined via an *in vitro* test, using methylviologen as an artificial electron donor and sodium dithionite for the reduction of methylviologen and of residual oxygen which would otherwise irreversible inhibit the hydrogenase. Hydrogen evolution of each well was measured by gas chromatography.
Using the screening assay, we tested the complete library and identified about 30 mutants (2 %) which were outstanding in the assay (Fig. 5), either by an enhanced or by a decreased hydrogenase activity. A specified analysis of these mutants by anaerobic adaptation of 50 ml culture finally resulted in one strain (*CM75*), that demonstrated a significantly decreased hydrogenase activity, indicating deficiencies in the induction of the hydrogenase or a loss of function of the enzyme itself. Control measurements with *CW 388*˙ revealed a usual wild type hydrogenase activity of about 120-150 nmol $H_2 \cdot h^{-1} \cdot \mu g\ Chl^{-1}$ whereas *CM75* produces only hydrogen gas with rates of 20-25 nmol $H_2 \cdot h^{-1} \cdot \mu g\ Chl^{-1}$ (Fig. 6). Northern Blot analyses of the hydrogenase mRNA showed a significantly lowered level of hydrogenase transcript (data not shown).

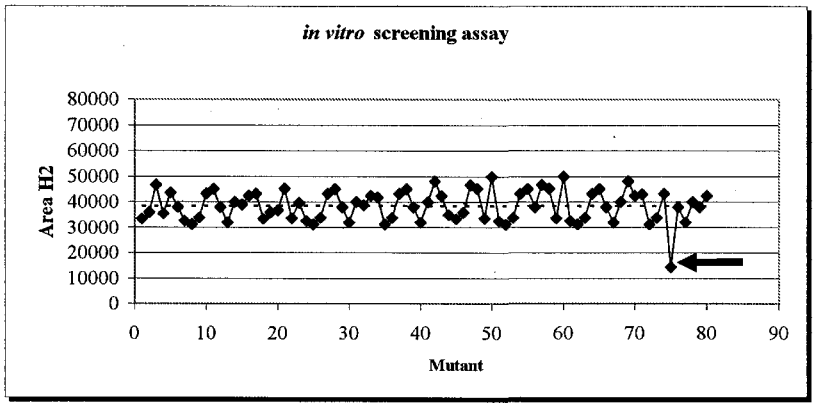

Figure 5. Diagram of one typical test serial.
The dotted line indicates the average H_2 area value of each deep well. The abscissa shows the number of the respective transformant, while the ordinate depicts the area value of the hydrogen peak that was obtained by gas chromatography. Mutant *CM75* (indicated by the black arrow) showed a significantly decreased hydrogen evolution activity.

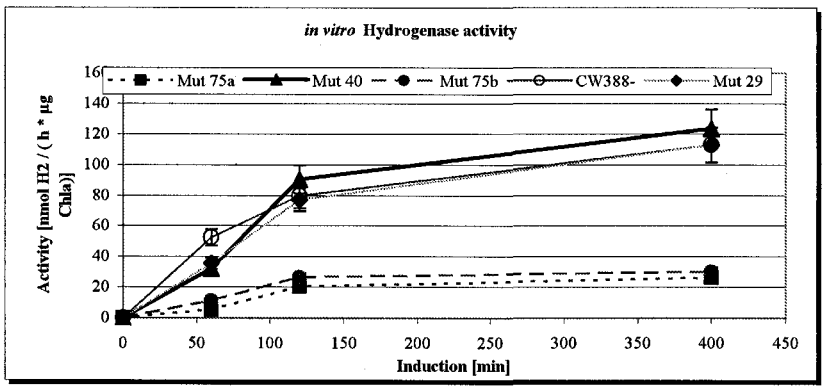

Figure 6. *In vitro* hydrogenase activity upon anaerobic adaptation.
Two cultures of *CM75* (a, b) one culture of *CW388*$^-$ and 2 cultures of core transformants that showed no significant increased or decreased hydrogen evolution activity in the deep well assay were bubbled with argon to obtain anaerobiosis. At the indicated time points of the anaerobic induction process samples were taken for the *in vitro* assay. Mutant *CM75* showed in both cases (a, b) only approximately 20 % of the activity measured in the control cultures.

DISCUSSION

Hydrogen production by green algae promises to be an ideal energy source for the future and the theoretical potential of [Fe]-hydrogenases for H_2-evolution is indisputable high [Melis and Happe, 2001]. Recently, it was observed that sealed cultures of sulfur-deprived *C. reinhardtii* cells develop a long lasting H_2-production activity [Melis et al., 2000]. But still the physiological activity of the up to now characterized algal strains has not reached the necessary efficiency for a biotechnological application [Happe et al., 2002].

The most problematic obstacles in this context are the limited *in vivo* production activities of the conventional algal cultures and the O_2 sensitivity of the known chlorophycean [Fe]-hydrogenases. Therefore it is important to find or develop alternative strains, with a higher physiological productivity and a reduced oxygen sensitivity. In this article we presented two different strategies which in combination could sooner or later lead us to this aim.

We described the identification and molecular biological characterization of the up to now only marginal examined alga species *C. moewusii* [Winkler et al., 2002b]. Its extraordinary high *in vitro* hydrogen production activity (Fig. 1) might either reflect a very efficient physiological integration of the [Fe]-hydrogenase in the anaerobic metabolism of *C. moewusii* or possibly argues for a very high specific enzyme activity. Two different *hydA* genes (*hydA1* and *hydA2*) were identified in the genome of *C.moewusii*. In both cases a major part of the corresponding cDNA was cloned and sequenced. First Northern blot experiments demonstrated that during anaerobic adaptation at least the transcription of *hydA1* corresponds to the development of the exceptionally high *in vitro* hydrogenase activity. The rapid gene expression correlates to the anaerobic gene regulation of *hydA* in *C. reinhardtii* [Happe and Kaminski, 2002].

The [Fe]-hydrogenase of green algae belong to a monophyletic group [Horner et al., 2002]. Phylogenetic examinations and alignment analyses with the *hydA* genes from *C. reinhardtii*, *S. obliquus* and *C. fusca* indicated that both *hydA* proteins of *C. moewusii* belong to a completely different subtype of chlorophycean [Fe]-hydrogenases. Corresponding to their different clade membership in the 18S SSU rRNA phylogeny of chlorophycean species [Proeschold et al., 2001], *C. reinhardtii*, the scenedesmacean species and *C. moewusii* carry each one of three different subtypes of green algal [Fe]-hydrogenases. Particularities like additional or displaced cystein residues or deviations in important regions of protein architecture indicate that the *HydA* proteins of *C. moewusii* belong to a new hydrogenase subtype. Therefore *C. moewusii* seems to be an interesting new candidate for further physiological examinations.

On the other hand we presented a new technique for the generation and identification of mutant strains with an altered physiological H_2 production activity. Therefore we generated and screened a mutant library which already in the start up phase enabled the identification of a mutant strain with an unusual hydrogen production activity. Further investigations of this mutant strain referring the genomic position of plasmid integration led to an interrupted gene that might affect the activity of the [Fe]-hydrogenase or the *hydA* expression rate (unpublished data). This demonstrates that the *in vitro* screening system is a reliable method to find mutants with differing hydrogen metabolism. Further examinations of hydrogenase defective mutants should provide important information about the physiological importance of the hydrogenase or the maturation process of the [Fe]-hydrogenase [Vignais et al., 2001]. Additionally, the described screening system enables the identification of mutants with an increased H_2-production activity.

For that purpose, interesting strains, which have been identified in the first stage of the screening system should be further analyzed not only by an *in vitro* test, but also by techniques which enable an exact determination of the respective *in vivo* activity. This

screening system should even allow the discovery of mutants which possesses an oxygen tolerant [Fe]-hydrogenase. Therefore the preliminary screening should be repeated under increasing O_2 concentrations.

One of the most important steps for the experienced application of this screening technique will be the change from a manual to an automated screening system. A high throughput screening was recently established in deep well plates for the creation of enantioselective biocatalysts [Jaeger et al., 2001]. According to this method, it should be possible to transfer the above described screening system to an industrial scale.

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IDENTIFICATION OF A *CIS*-ACTING ELEMENT CONTROLLING ANAEROBIC EXPRESSION OF THE *HYDA* GENE FROM *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

In *Chlamydomonas reinhardtii*, the expression of the *hydA* gene encoding the [Fe]-hydrogenase is controlled on the transcriptional level under anaerobic conditions. We have isolated a DNA fragment of 1819 bp upstream of the *hydA* transcription start site by Genome Walking. To understand the mechanism that underlies the anoxic regulation in *C. reinhardtii*, we have fused the promoter region of *hydA* to the arylsulphatase (*ars*) reporter gene lacking its own promoter. Several deletions within the region -1819 to-21 were introduced into a cell-wall deficient alga strain. Transformants expressing the Ars enzyme in sulphur sufficient medium were isolated and shown to transcribe the *hydA/ars* construct only under anaerobic conditions. The expression of the *ars* gene was high enough to allow quantitative measurements. The results indicate that the region between positions -128 and -21 with respect to the transcription start site is required for anaerobic specific gene expression and possibly contains at least one enhancer element.

KEYWORDS:

Anaerobic adaptation, [Fe]-hydrogenase, hydrogen evolution, green algae, promoter

INTRODUCTION

Chlamydomonas reinhardtii, a unicellular green alga, is capable to produce molecular hydrogen in response to anaerobic stress conditions. By switching the oxidative pathway to a fermentative metabolism, the evolution of hydrogen plays an important role in anaerobic energy metabolism to reoxidize accumulated redox equivalents [Happe et al., 2002; Melis and Happe, 2001; Vignais et al., 2001]. From *C. reinhardtii*, a monomeric [Fe]-hydrogenase

with high specific activity was isolated and biochemically characterized [Happe and Naber, 1993]. The oxygen-sensitive enzyme of 48 kDa contains a short transit peptide of 56 amino acids which routes the hydrogenase to the chloroplast stroma [Happe et al., 1994].
The key enzyme hydrogenase catalyses the reversible reduction of protons to molecular hydrogen. Inhibitor experiments indicate that the ferredoxin PetF functions as natural electron donor linking the hydrogenase to the photosynthetic electron transport chain [Florin et al., 2001].

Recently, the isolation and characterization of the hydrogenase gene *hydA* from *C. reinhardtii* was published [Happe and Kaminski, 2002]. The cDNA sequence comprises a length of 2399 bp and the coding region of the genomic DNA includes seven introns with sequences at their 5` and 3` ends corresponding to the typical splicing sequences from eukaryotes [Breathnach and Chambon, 1981]. The transcription start site 158 bp upstream of the ATG start codon was determined by RACE-PCR. Northern blot analysis demonstrates that the transcription of the *hydA* gene is rapidly induced during anaerobic adaptation of the cells. On the basis of this study, we have recently isolated and characterized the *hydA* genes of the green algae *Scenedesmus obliquus* and *Chlorella fusca* which also encode oxygen-sensitive [Fe]-hydrogenases [Florin et al., 2001; Winkler et al., 2002].

As described for other green algae genes, the region upstream of the transcription start position of the *hydA* gene does not contain a highly conserved TATA-box element or any other known general transcription motifs [Happe and Kaminski, 2002]. Although the expression of the *hydA* genes from different green algae is dependent on oxygen deficiency [Happe and Kaminski, 2002; Florin et al., 2001; Winkler et al., 2002], nothing is known about conserved DNA sequences in the promoter regions mediating anaerobic-specific gene expression of green algae. The promoter regions also lack conserved *cis*-acting elements from genes encoding anaerobic stress proteins in higher plants like GT or GC motifs [Olive et al., 1991; Walker et al., 1987].

In this work, we identified a *cis*-acting element associated with the *hydA* promoter of *C. reinhardtii* by introducing reporter gene fusions carrying various *hydA* 5` upstream regions into *C. reinhardtii*. Our results indicate that a sequence contained within the -128 to -21 region of the *hydA* promoter is sufficient to confer anaerobic specific gene expression.

MATERIALS AND METHODS

Strains, culture conditions and anaerobic adaptation

The *Chlamydomonas reinhardtii* host used in this study is the cell-wall deficient CW ⁻388⁻ (cw15 arg7 nit1 mt^+) strain lacking argininosuccinate lyase [de Hostos et al., 1989]. The cells were grown photoheterotrophically [German and Levine, 1965] on solidified agar medium or in batch cultures at 25°C under continuous irradiance of 150 µmol photons·m^{-2}·s^{-1}. The routinely used medium was Tris-acetate-phosphate (TAP) medium containing 200 µg arginine /ml. Liquid cultures were bubbled vigorously with air supplemented with 5 % CO_2. After harvesting the cells by centrifugation (8 min, 5000×*g*) in the mid-exponential growth phase (1×10^6 cells/ml), the pellet was resuspended in 0.1 vol of fresh TAP medium and the algae were anaerobically adapted by flushing the culture with argon in the dark. For anaerobic adaptation, cells on agar plates were incubated in a chamber filled with a gas mixture of N_2 and H_2.

Hydrogen evolution assay

The *in vitro* hydrogenase activity was determined by using a gas chromatograph (Hewlett Packard 5890 A Series II, column: molecular Sieve 5 A, Mesh 60/80). As described before [Happe and Naber, 1993], methylviologen reduced by sodium dithionite was used as electron donor. One unit is defined as the amount of hydrogenase evolving 1 μmol $H_2 \cdot min^{-1}$ at 25°C.

Genome Walking with genomic DNA

Applying the Universal GenomeWalker™ Kit (CLONTECH), genomic libraries from *C. reinhardtii* were generated by digestion with different blunt end cutting endonucleases (*Hin*cII, *Dra*I, *Stu*I, *Sca*I and *Sma*I) and by adapter ligation at the ends of the resulting DNA fragments. These libraries were used in a first polymerase chain reaction (PCR) [Siebert et al., 1995] in which a gene-specific primer derived from the DNA sequence upstream of the transcription start site of the *hydA* gene of *C. reinhardtii* was used in combination with the adapter primer (AP1). In a secondary PCR, 1 μl of the first PCR served as a template using a nested gene-specific primer along with the nested adapter primer (AP2). The resulting products were cloned into pGEM® T-Easy and sequenced by the didesoxy chain termination method [Sanger et al., 1977].

Construction of chimeric constructs

For promoter deletions, the respective regions from the *hydA* promoter of *C. reinhardtii* were amplified by PCR using upstream oligonucleotides with a *Sal*I restriction site at their 5` ends and a downstream oligonucleotide with a *Kpn*I restriction site at the 5` end. The PCR products have had different promoter regions (1819 to 20 bp) upstream of the transcription start site and 44 bp of the 5`UTR of the *hydA* gene. After *Sal*I/*Kpn*I double digestion, the DNA fragments were cloned into the *Sal*I/*Kpn*I sites of Plasmid pJD54 containing the arylsulphatase (*ars*) gene without a promoter [Davies and Grossman, 1994]. The *Kpn*I and *Sal*I sites upstream of the *ars* gene are unique. The resulting plasmids pMR14, pMR16, pMR19, pMR21, pMR23 and pMR30 contain chimeric gene constructs bearing deleted promoter regions of the *hydA* gene upstream of the promoterless *ars* gene.

Nuclear transformation of C. reinhardtii

Cells of *C. reinhardtii* were transformed by agitation in the presence of glass beads (1.0 mm) [Kindle, 1990]. The *C. reinhardtii* culture was grown in liquid TAP medium containing 200 μg arginine /ml up to a density of 2×10^6 cells/ml. After centrifugation (5 min, 5000×*g*), the pellet was resuspended in 0.1 vol liquid TAP medium lacking arginine and incubated for 30 min at 20°C. The transformation mixture contained 0.3ml resuspended cells, 0.1 ml 20 % (w/v) polyethylene glycol, 1 μg pARG7.8 (a plasmid bearing *arg7* gene [Debuchy et al., 1989; Purton and Rochaix, 1994] and 2 μg of the co-transforming chimeric promoter constructs. After shaking, the supernatant was transferred to TAP agar plates lacking arginine. Routinely, several hundred transformants were recovered.

Assays of arylsulphatase activity

The arylsulphatase (Ars)-activity in transformants growing in the absence of exogenously supplied arginine was detected qualitatively by screening colonies on plates for their ability to hydrolyze the chromogenic sustrate 5-bromo-4-chloro-3-indolyl-sulphate (X-SO_4) according

to Davies et al. (1992). Quantitatively, Ars-activity was spectrophotometrically measured as a change in absorbance at 650 nm in the supernatant of 1×10^6 cells using X-SO_4 [Lien and Schreiner, 1975].

Isolation of nucleic acids and genetic analyses

Genomic DNA from transformed *C. reinhardtii* cultures was isolated by lysing cells in 2 % SDS, 400 mM NaCl, 40 mM EDTA and 100 mM Tris-HCl pH 8.0. After phenol extraction, the DNA was precipitated and resuspended in water. Digested genomic DNA was separated on 1 % agarose gels, transferred to nylon membranes (Hybond, Amersham), and hybridized with a 1020 bp promoter DNA fragment containing the region upstream of the transcription start site. To analyze genomic DNA by PCR, a rapid method for screening a large number of colonies on plates was developed. Cells were scraped off from plates and resuspended in 10 mM Tris-HCl, 10 mM EDTA and 150 mM NaCl. After disrupting the cells (Cell disruptor B 15, Branson), 2 µl of the resulting suspension can be used directly in the PCR.

Total RNA was isolated from algae grown under aerobic conditions and after anaerobic adaptation according to the method of Johanningmeier and Howell (1984). Northern blot analysis was performed as described by Sambrook et al. (1989). RNA, transferred to nylon membranes (Hybond$^+$, Amersham) was hybridized with RNA probes, which were labeled with digoxygenin (DIG)-dUTP by *in vitro* transcription. Transcripts of the *hydA* gene were detected using a 1.0 kb *Sma*I cDNA fragment whereas transcripts of the *ars* gene were detected using a 0.2 kb cDNA fragment.

RESULTS

Nucleotide sequence of the hydA promoter region

The genomic nucleotide sequence of the *C. reinhardtii hydA* gene was previously published and sequenced up to 1020 bp upstream of the transcription start site [Happe and Kaminski, 2002]. We used the Genome Walking method to characterize a 799 bp DNA fragment further upstream of the 1020 bp *hydA* promoter fragment. The whole DNA sequence of 1819 bp upstream of the *hydA* transcription start site was used to create chimeric *hydA/ars* gene constructs. The mosaic structure of the *hydA* gene and the 5`upstream region is shown in Figure. 1A. The nucleotide sequence data reported in this study is available from the EMBL, GenBank and DDBJ nucleotide sequences databases under accession no. AJ308413.2.

In an effort to identify *cis*-acting elements involved in anaerobic transcriptional regulation of the *C. reinhardtii hydA* gene, a series of 5`nested deletions produced by PCR were fused to the promoterless *ars* reporter gene. The arylsulphatase is a periplasmic enzyme that is secreted into the surrounding medium by cells lacking a cell wall [de Hostos et al., 1989; Lien and Schreiner, 1975]. Ars activity is easily assayed in colonies using the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulphate (X-SO_4); the algae colonies expressing arylsulphatase become blue on agar medium. [Davies et al., 1992]. No *ars* activity or *ars* mRNA is detectable in untransformed cells grown in sulphur-sufficient medium [de Hostos et al., 1989]. Chimeric *hydA/ars* gene constructs were introduced into *C. reinhardtii* mutants by the glass bead method according to Kindle (1990). Six chimeric *hydA/ars* gene constructs pMR14, pMR16, pMR19, pMR21, pMR23 and pMR30 were generated that contain parts of the upstream region up to positions -1819, -474, -340, -238,

-128 and -20 with respect to the transcription start site (Fig. 1B). The 3`end of the *hydA* DNA fragment was always at position +44. After co-transformation with plasmid pARG7.8 carrying the *arg7* wild-type gene of *C. reinhardtii* [Debuchy et al., 1989], mutants of *C. reinhardtii* lacking argininosuccinate lyase were complemented and able to grow on TAP medium in the absence of arginine (arginine prototrophs).

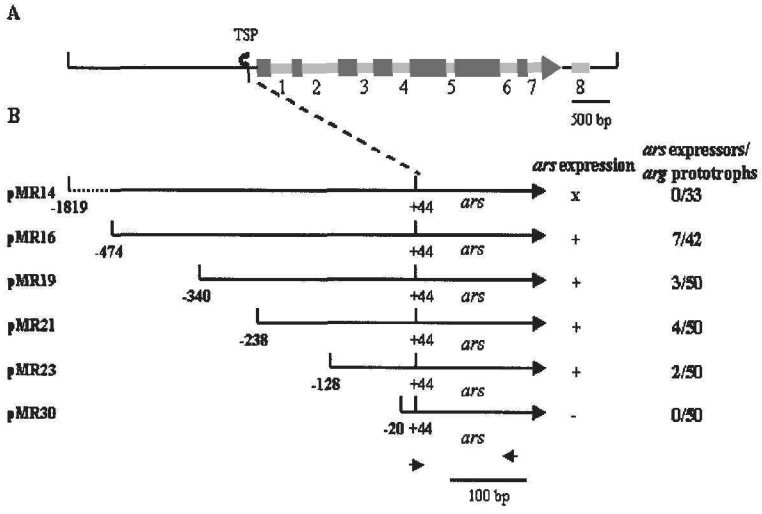

Figure 1. Schematic map of the genomic DNA region of *hydA* and of chimeric *hydA* promoter constructs.

(A) The mosaic structure of *hydA* is indicated by gray (exons) and white (introns) boxes. TSP, transcription start point. (B) A series of 5`nested deletions of the -1819 to -20 *hydA* promoter fragment was fused to the promoterless *ars* gene in pJD54 and introduced into the *C. reinhardtii* strainlacking argininosuccinate lyase by co-transformation with pARG7.8. Numbering is relative to the transcription start site of the *hydA* gene. Arginine prototrophs were analyzed with the qualitative X-SO_4 assay. (+) and (-) indicate exhibiting or not exhibiting arylsulphatase activity. The presence of the chimeric construct in each *ars*-transformant was verified by PCR using an upstream oligonucleotide that hybridizes to the *hydA* promoter fragment and a downstream oligonucleotide that hybridizes to the *ars* gene asindicated with black arrows at the bottom.
All mutants transformed with the chimeric construct pMR14 lack the *hydA* promoter region upstream of the promoterless ars gene in the genome of the *C. reinhardtii* mutants. (x) indicates that no *ars*-expression (0/33) was detected as a result of the absence of the chimericconstruct in the genome of these mutants. Transformants expressing *hydA/ars* are listed together at the right site with the number of transformants tested.

PCR and Southern blot analysis of transformants

The co-transformation rate was analyzed by PCR using an oligonucleotide derived from the *hydA* promoter sequence in combination with an oligonucleotide derived from the *ars* gene (Fig. 1B). We developed a method for screening colonies on plates rapidly by using PCR without previous isolation of genomic DNA. Disrupting cells in the presence of EDTA makes genomic DNA available for PCR analysis. Although between 4 and 16 % of arginine prototrophs exhibited arylsulphatase (Ars)-activity in sulphur-supplemented medium, PCR analyses revealed that more than 30 % of arginine prototrophs were co-transformed (data not shown). Colonies that were screened positive by using PCR were tested subsequently by Southern hybridization to verify that they indeed contained at least one copy of an intact chimeric construct. The most *ars* expressing co-transformants contained a single copy of the deletion construct whereas two or more copies in the genome of co-transformed cells were very rare (data not shown).

Qualitative and quantitative arylsulphatase assay of transformants

Northern blot analysis demonstrated that the *hydA* gene is expressed rapidly after the beginning of anaerobic adaptation [Happe and Kaminski, 2002]. Therefore, arginine prototrophs on agar plates were screened for Ars-activity by using the qualitative X-SO_4 assay after an anaerobic adaptation of at least 3 h in a chamber filled with N_2 and H_2. Initially, colonies on plates were grown for 7 days under aerobic conditions. After dropping X-SO_4 on colonies, co-transformants can be identified by a blue staining in the surrounding medium that appeared within 6-12 h (Fig. 2A). Anaerobic conditions were verified by measuring the *in vitro* hydrogenase activity of the colonies with reduced methylviologen.

Interestingly, a weak Ars-activity was also detected (Fig. 2B) in the same colonies incubated over a longer period under aerobic conditions. In all experiments only these colonies exhibited Ars-activity that carried the chimeric *hydA/ars* gene construct. Therefore, expression of the endogenous *ars* gene caused by local sulphur depletion can be excluded. When screening colonies on plates after a growth of 4 days, a time in which cells did not exhibit hydrogenase activity, co-transformants did not express the *ars* reporter gene under the control of the *hydA* promoter even during anaerobic adaptation. Probably, a higher cell density is necessary to get a visible blue staining. We concluded that colonies on plates become oxygen depleted within 7 days as a consequence of respiratory activity. Reasonable, after a few days cells on plates get such a density that only the outer cells of the colonies show photosynthetic activity. Quinn et al. (2002) reported that heterotrophic cultures become oxygen depleted measured with standardized oxygen electrode when they are suspended by slow basal stirring. Therefore expression of the *hydA* gene is probably induced in colonies grown on plates caused by microanaerobic conditions. *C. reinhardtii* cells on agar plates become anaerobic in a few days during normal growth conditions. As a consequence, expression of genes involved in response to anaerobic stress conditions is physiologically relevant during aerobic growth in nature and even in laboratory.

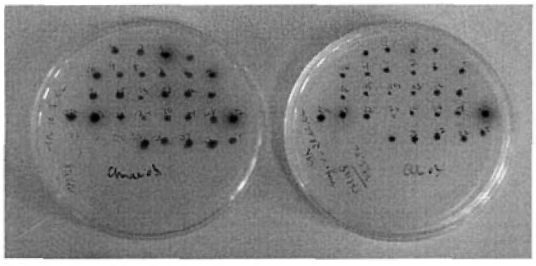

Figure 2. Detection of Ars-activity in arginine prototrophs during anaerobic or aerobic adaptation.
Arginine prototrophs were screened for Ars-activity by using the qualitatively X-SO_4 assay. After growing cells on plate during anaerobic (A) or aerobic (B) adaptation, X-SO_4 was dropped on colonies of strain MR16 generated by transformation with plasmid pMR16.

Activation of *hydA* expression exclusively under anaerobic or microanaerobic growth conditions of *C. reinhardtii* cells was verified by the quantitative X-SO_4 assay. As shown in Figure 3A, Ars-activity can be detected after an anaerobic adaptation by bubbling co-transformants in liquid medium with argon within a few days. In contrast to the results of the qualitative X-SO_4 assay, co-transformants in liquid medium incubated in the presence of oxygen did not express the *ars* reporter gene. Furthermore, we examined the time course of anaerobiosis-specific induction by measuring the Ars-activity in the supernatant spectrophotometrically as a change in absorbance at 650 nm (Fig. 3B). There is a continuous increase in Ars-activity of an individual co-transformant within 5days of anaerobic adaptation. No Ars-activity was obtained in the recipient strain.

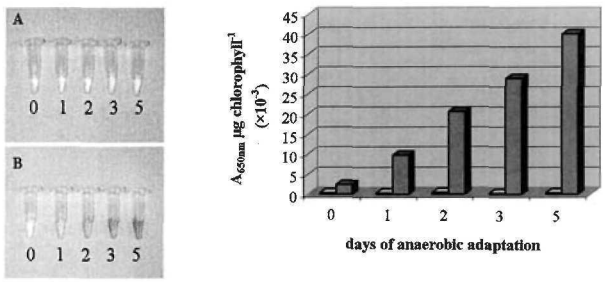

Figure 3. Time course of anaerobic induction of Ars-activity.
At different times of anaerobic adaptation cells were pelleted and Ars-activity was measured spectrophotometrically in the supernatant as a change in absorbance at 650 nm using X-SO_4 as substrate. Strain MR16 carrying the chimeric *hydA*/*ars* construct pMR16 and the recipient strain of *C. reinhardtii* were tested.
(A) The recipient strain of *C. reinhardtii* did not exhibit Ars-activity in sulphur-supplemented medium. Numbers at the bottom indicate days of anaerobic adaptation.
(B) The blue staining in the supernatant of strain MR16 becomes visible after 1 day.
(C) A_{650nm} values of the recipient strain (open bars) and the MR16 strain (closed bars) were normalized to the cell density of the culture.

Northern analysis of transformants under anaerobic conditions

To verify the presence of *ars* mRNA, we hybridized total RNA with the *ars* specific RNA probe from an individual co-transformant grown in sulphur sufficient medium. As shown in lane 2 of Figure 4A, a weak signal was detected after 1 h of anaerobic adaptation. In the presence of oxygen no *ars* transcript was detected in co-transformed cells carrying the *ars* reporter gene under the control of the *hydA* promoter (Fig. 4A, lane 1). These results correlate with Northern anlysis of the *hydA* transcript. No *hydA* transcript could be detected before adaptation (Fig. 4B, lane 1), but a significant signal occurred after 1 h of anaerobiosis (Fig. 4B, lanes 2-3). Happe and Kaminski (2002) pointed out that *hydA* expression is induced rapidly after 15 min of anaerobic adaptation. As seen in Figure 4, the *hydA* transcript is more abundant than the *ars* transcript, although both genes are regulated by the same promoter.

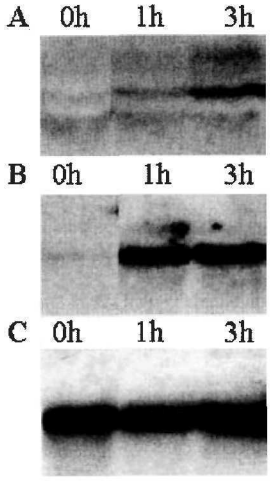

Figure 4. Northern blot analysis of transformants.
C. reinhardtii cells of strain MR16 were anaerobically adapted by flushing the culture with argon in the dark. Adapted cells were harvested at 0 h, 1 h, and 3 h, and total RNA was isolated as described in Materials and Methods. Northern hybridization with the *ars* specific probe (A) and the *hydA* specific probe (B) are shown.
(C) The same RNA was hybridized with a constitutive expressed gene (*atpD*) as control.

A 108 bp region between -128 and -21 conferring anaerobic expression of the hydA gene

As shown in Figure 1B, all arginine prototrophs resulted from co-transformation with pMR14 (-1819 to +44 of the *hydA* promoter region) did not exhibit Ars-activity. This is presumably the consequence of the lack of the chimeric construct in their genome indicated by PCR analyses of all resulting arginine prototrophs.

Strains MR16, MR19, MR21 and MR23 harbouring constructs with deletions up to

position -128 (Fig. 1B) permitted expression of the *ars* reporter gene indicating that the 172 bp region between -128 and +44 was sufficient for anaerobic responsive regulation. In control experiments, we introduced the promoterless *ars* gene into *C. reinhardtii*. No *ars* expressing transformants were detected indicating that positional effects as a result of integration of the *ars* gene near another promoter in the genome does not occur frequently. However, additional deletion up to position -20 resulted in a total loss of Ars-activity in sulphur supplemented medium (Fig. 1B, strain MR30 harbouring construct pMR30) although PCR analysis indicated that 15 of 50 arginine prototrophs contained indeed the chimeric *hydA/ars* gene construct in their genome. This suggests that at least one *cis*-acting element lies between position -128 and -21 relative to the transcription start site of *hydA* conferring anaerobic-specific expression. Probably, the *cis*-acting element in the region between -128 and -21 functions as enhancer, that activates transcription in response to oxygen depletion. However, further experiments are necessery to determine whether this *cis*-acting element functions as a transcriptional silencer or enhancer.

DISCUSSION

In order to localize *cis*-acting sequences in the *hydA* promoter region, chimeric *hydA/ars* gene constructs were generated and introduced into *C. reinhardtii* recipient strain. The transformants containing the chimeric constructs expressed the *ars* gene under the control of the *hydA* promoter. PCR analysis indicated that deleted *hydA* promoter regions did not change their localization as a consequence of recombination processes and that they were still localized upstream of the promoterless *ars* gene. Therefore, in transformants *ars* expression is controlled transcriptionally by the *hydA* promoter meaning that the endogenous *hydA* gene is regulated at the level of transcription in agreement with most of the nuclear genes of *C. reinhardtii* [Hahn et al., 1996].

Northern analyses of the *hydA* and *ars* transcript in co-transformants as well as the quantitative Ars-assay indicate that oxygen depletion causes transcriptional activation of the *hydA* gene. However, the accumulation of the *hydA/ars* transcript is low relative to the endogenous *hydA* gene (Fig. 4A and B). A similar result was also reported by Davies et al. (1992). The amount of *tubB2/ars* transcript is at least 10 fold less in contrast to the endogenous *tubB2* transcript. The authors speculate that the chimeric transcripts may be synthesized at a lower rate or that the primary transcript may not be efficiently processed to the cytoplasm of the cell. Possibly, the transcription of the chimeric *hydA/ars* gene constructs was also limited because an enhancer element around the *hydA* gene was not included.

The qualitatively Ars-assay has enabled us to identify a *cis*-acting element in the promoter region between -128 and -21. The 108 bp region without any promoter elements like TATA- or CAAT-box is sufficient to confer anaerobic activation of the *hydA* gene expression. No homology was found comparing this region with upstream regions of other anaerobic inducible genes of higher plants. For example, the GT box (GGTTT) in the promoter regions of the maize *gapC4* and *adh1* genes plays an important role in anaerobic specific gene expression [Ferl, 1990; Geffers et al., 2000; Olive et al., 1991]. Further *cis*-sequences (GT or G boxes) are involved when higher plants are exposed to anoxic stress [Walker et al., 1987; de Vetten and Ferl, 1995].

Very recently, it was shown that the three target genes *cyc6*, *cpx1* and *crd1* of the copper deficiency signal transduction pathway are also activated during anaerobic adaptation in *C. reinhardtii* [Quinn et al., 2002]. Accumulation of these transcripts can be observed within one day when cultures of *C. reinhardtii* are suspended by slow basal stirring under

normal room lighting as a result of oxygen deficiency caused by respiratory activity in the presence of acetate. The same genes are activated also in response to copper-deficiency by copper-response (CuRE) elements that function as target sites for a transcriptional activator [Quinn et al., 2002]. However, the CuRE elememts are necessary but not sufficient for transcriptional activation of *cpx1* under anaerobic conditions, which requires also a hypoxia-response element (HyRE). The transacting master regulator (CRR1) which is required for the oxygen and copper deficiency was isolated [Quinn et al., 2002]. The authors speculate that oxygen-and copper-deficiency responses share signal transduction components.

However, the expression of the hydrogenase gene *hydA* is independent of copper and CRR1 which suggests that a second putative oxygen deficiency signaling pathway exists in *C. reinhardtii*. In this study we present first hints (sequence elements) for this induction pathway that induce the transcription of *hydA*.

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GLYCOLIPID LIQUID CRYSTALS AS NOVEL MATRICES FOR MEMBRANE PROTEIN MANIPULATIONS.

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ABSTRACT

We have developed novel synthetic glycolipids, 1,3-di-o-phytanyl-2-o-(glycosyl) glycerols bearing maltooligosaccharide headgroups, $Mal_N(Phyt)_2$, (the number of glucose units N = 1, 2, 3, and 5). Their values of gel-liquid crystalline phase transition temperature, T_m are below 0°C, making the Mal_N $(Phyt)_2$ considerably easier to handle than conventional glycolipids. The lamellar-forming lipid, Mal_3 $(Phyt)_2$, is particularly suitable for preparing vesicles and planner lipid bilayer membranes that exhibit a significantly lower H^+ permeability as compared to that of egg yolk phosphatydiylcholine (EPC). Moreover, the oxygen evolution activity of thermophilic cyanobacterial photosystem II complex (PS II) reconstituted into the Mal_3 $(Phyt)_2$ vesicles is significantly higher than that reconstituted into phospholipid-vesicles such as EPC and diphytanoyl-phosphatidylcholine (DPhPC). The present results indicate the usefulness of synthetic glycolipids for manipulating membrane proteins, e.g., the reconstitution of membrane proteins.

INTRODUCTION

Lyotropic lamellar (L_α) liquid crystals (LC), in a form of vesicle or planar membrane, are important for membrane research to elucidate both functional and structural aspects of membrane proteins. Membrane proteins so far investigated are receptors, substrate carriers, energy-transducting proteins, channels, and ion-motivated ATPases [1-11]. The L_α liquid crystals have also been proved useful in the two-dimensional crystallization of membrane proteins[12, 13], in the fabrication of protein micro-arrays[14], and biomolecular devices[15]. Usefulness of an inverted cubic LC in the three-dimensional crystallization of membrane proteins has also been recognized[16].

To date, the lipids so far used have been mainly extracts from natural sources such as EPC and archaeal lipids[17,18] The chemical stability of EPC, however, is not sufficient and the membrane permeability to H^+ is sometimes too high for quantitative analyses of membrane protein functions. Though archaeal lipids display many preferable features for

handling membrane proteins, e.g., formation of monolayers (tetraethers) or bilayers (diethers) with low H^+ permeability[19], low hydrated solid liquid-crystalline transition temperatures[20, 21], they are not readily available in amount and are often complex mixtures of heterogeneous lipids, making control of the membrane properties difficult. In this respect, synthetic lipids that are available in a chemically pure form are much preferable.

With an aim to develop synthetic lipids for the membrane protein manipulation, we here propose synthetic glycolipids, $Mal_N(Phyt)_2$, whose chemical structures are shown in Figure 1[22, 23]. $Mal_N(Phyt)_2$ is a class of a recently developed glycolipid family with isoprenoid-type hydrophobic chains[22-27] The phytanyl chain consists of highly branched 3,7,11,15-tetramethylhexadecyl group (a total of 20 carbon atoms) and is a common basic core structure of major lipids of archaebacterial plasma membranes[28]. The phytanyl chains are linked to the hydrophilic groups by an ether bond. Compared with an ester bond, the ether linkages are obviously more stable to hydrolysis at high temperatures and high and low values of pH. We choose sugars as their headgroups, since sugars are empirically known to stabilize protein functions and structures in aqueous solutions[29].

In this article, we describe 1) general features of physical properties of aqueous $Mal_N(Phyt)_2$ and 2) the use of $Mal_3(Phyt)_2$ for functional reconstitution of thermophilic cyanobacterial photosystem II complex (PS II), one of the key elements for a biomolecular device for hydrogen production[15]. PS II performs a series of photochemical reactions consisting of adsorption and transfer of light energy, charge separation, abstraction of electrons from water molecule by the positive charge resulting in molecular oxygen evolution, and reduction of plastoquinone by the negative charges[30]. PS II has the most complex structure among the other protein complexes in thylakoid, e.g., cyanobacterial PS II is composed of most presumably more than 15, subunits[30]. PS II, however, is relatively labile; readily loses its water splitting activity owing to loss of the Mn-cluster from the complexes[31]. The stabilization of PS II under engineering conditions is therefore prerequisite for fabricating the biomolecular device for hydrogen production.

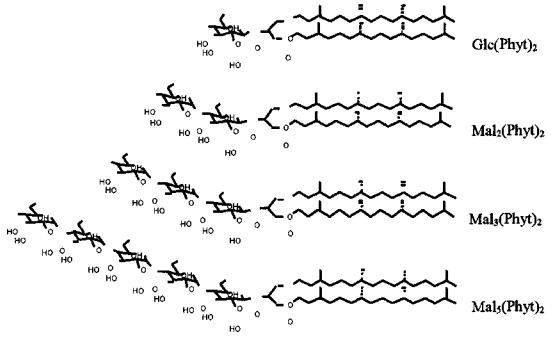


Figure 1. Chemical structures of 1,3-di-o-phytanyl-2-o-(β-glycosyl) glycerols bearing maltooligosaccharide headgroups, $Mal_N(Phyt)_2$.
N: The number of glucose units in the headgroup.
A phytanyl chain contains 16 carbon atoms in the main chaim and 4 branched methyl groups.

RESULTS

Physical properties of $Mal_N(Phyt)_2$/water systems -Phase behavior and characterstics of the bilayer membranes-

One important character of $Mal_N(Phyt)_2$ is their low gel-LC transition temperature, T_m. In DSC thermograms of aqueous $Mal_N(Phyt)_2$, neither endothermic nor exothermic peak associated with phase transition of the lipid appears over the temperature range from -100°C to 100°C[22]. These considerably lower values of T_m are in marked contrast to those of conventional straight-chained glycolipids. T_m values for the conventional glycolipids with $C_{16} \sim C_{18}$ chains are generally significantly higher than room temperature[32].

The $Mal_N(Phyt)_2$/water systems are thus always in a fluid state at temperatures above the freezing point of water, 0°C. Moreover, as seen from the phase diagrams of the $Mal_N(Phyt)_2$/water systems (Fig. 2), the LC structures of $Mal_N(Phyt)_2$ can be readily controlled by changing the number of glucose residues N in the headgroup; an inverted micellar cubic phase of Fd3m symmetry for $Glc(Phyt)_2$ (N=1)[23], an H_{II} phase for $Mal_2(Phyt)_2$ (Fig. 2a), and an L_α phase for $Mal_3(Phyt)_2$ (Fig. 2b) and $Mal_5(Phyt)_2$ (Fig. 2c)[22]. It is also noted that the LC phase structures are practically temperature-independent at least between 0 and 80°C.

Permeability of water soluble species through bilayer membranes of $Mal_N(Phyt)_2$ is significantly smaller than that through PC membranes. Figure 3 compares temperature effects on permeability of calcein through bilayr membranes of $Mal_5(Phyt)_2$ and DPPC[33]. The permeability through the $Mal_5(Phyt)_2$ membrane is significantly smaller and less temperature dependent as compared to that through the DPPC membrane. This may reflect the temperature insensitive nature of the L_α phase structures as inferred from the phase diagram (Fig. 2c). We also noted that the L_α-forming lipid, $Mal_3(Phyt)_2$, gives vesicles or planner bilayer membranes that exhibit a significantly lower H^+ permeability compared to that of EPC[33,34]. Moreover, it appears that the planar lipid bilayer of $Mal_3(Phyt)_2$ is considerably stable; it was usually stable for at least 2-3 days, whereas soybean phospholipid bilayers formed under the same conditions were usually stable for less than 8 h[34]. $Mal_5(Phyt)_2$, on the other hand, did not give a stable planar bilayer, most presumably due to a larger cross section area of the headgroup as compared to that of the hydrophobic chain[34,35]. Taking all these results together, we conclude that $Mal_3(Phyt)_2$ is most promising as matrices for membrane proteins.

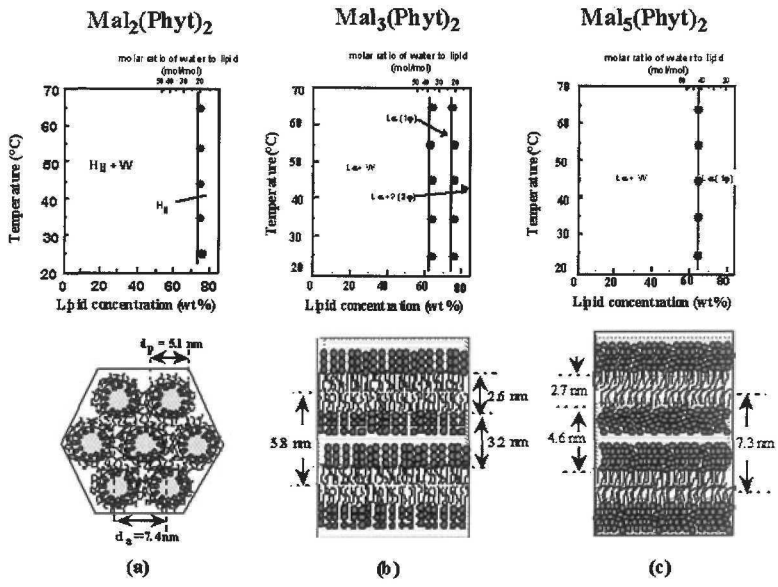


Figure 2. The partial phase diagram of $Mal_2(Phyt)_2$/water (a), $Mal_3(Phyt)_2$/water (b),and $Mal_5(Phyt)_2$/water (c) systems, respectively. The lower traces represent the schematic structure of an H_{II} phase of the $Mal_2(Phyt)_2$/water system, an L_α phase of the $Mal_3(Phyt)_2$/water and $Mal_5(Phyt)_2$/water systems, respectively.

Figure 2c. The partial phase diagram of $Mal_3(Phyt)_2$/water system.
This system was examined up to 82 wt % lipid.1φ: One-phase region an L_α phase. 2φ: Two-phase region (L_α+ dilute aqueous lipid)
The mesophase structure that coexists with the $L_\alpha$phase above 75 wt % lipid is unknown.

Figure 2c. Schematic representation of the structure of an L_α phase of the $Mal_3(Phyt)_2$/water system.

Figure 2d. The partial phase diagram of This system was examined up to 82 wt % lipid.
1φ: Single-phase region of an $L_\alpha$phase that extends at least up to 82 wt %.
2:φ Two-phase region (L_α + dilute aqueous lipid).

Figure 2d. Schematic representation of the structure of an L_α phase of the $Mal_5(Phyt)_2$/water system.

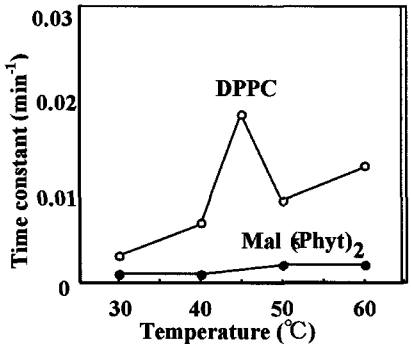


Figure 3. Temperature effects on permeability of calcein through lipid bilayer membranes of $Mal_5(Phyt)_2$ and DPPC.

Functional reconstitution of thermophilic cyanobacterial photosystem II complex (PS II). Incorporation of PS II into vesicles.

Purified PS II that was isolated from thermophilic cyanobacterium *Synechococcus elongatus* grown at 55°C was supplied by Dr. M. Hirano at Toray Research Center, Inc. (TRC), Kamakura. Oxygen-evolving activity of PS II at 40°C was 600 - 1900 μmol O_2/mg Chl*a* $\cdot h^{-1}$. PS II incorporation into lipid vesicles was possible only when PS II was incubated with a solubilized lipid with n-octyl-β-D-glucoside (OG). Thus, a mixture of $Mal_3(Phyt)_2$ and sulphoquinovosyldiacylglycerol, SQDG, (9:1, mol/mol) was solubilized with OG in a Mes-Hepes/betaine buffer (1 mM Mes/1mM Hepes/10 mM NaCl/5 mM $MgCl_2$/1 M betaine). PS II was then added to the solubilized lipid to give a final PS II concentration of 8μg Chl*a* /ml of the Mes-Hepes/betaine buffer containing 2 mM lipid, 0.22 mM SQDG, and 40 mM OG. The solubilized lipid/SQDG/OG/PS II mixture was then dialyzed against 100-fold volume of the Mes-Hepes/betaine buffer containing Bio-Beads SM-2 at 4°C for 12 h under dark. We confirmed that more than 90 % of the PS II was incorporated into the lipid vesicles. Lipids examined are two glycolipids, $Mal_3(Phyt)_2$, digalactosyldiacylglycerol (DGDG) and a range of phospholipids, e.g., EPC, palmitoyloleoyl-PC (POPC), and dioleoyl-PC, diphytanoyl-PC (DPhPC). Diameters of the PS II incorporated vesicles were 90 ± 20 nm, 130 ± 25 nm, 130 ± 35 nm, 115 ± 28 nm, 140 ± 42 nm, and 140 ± 50 nm for $Mal_3(Phyt)_2$, DGDG, EPC, POPC, DOPC, and DPhPC, respectively.

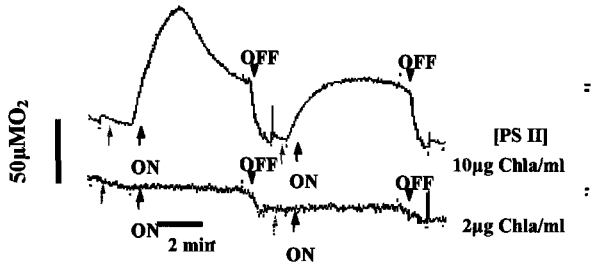
Oxygen evolution activity of reconstituted PS II.

Typical example of light-induced O_2 evolution of reconstituted PS II/$Mal_3(Phyt)_2$ system with different PS II concentrations is shown in Figure 4a. In this experiment, we add an 8μl of 100 mM DCBQ as an electron acceptor just before each ON-OFF cycle. When the actinic light is removed, we simultaneously flash the cell by bubbling air to remove evolved oxygen quickly reequilibrating the oxygen level of the reaction mixture. For the first ON-OFF cycle of the upper trace (10μg Chl*a*/ml), the O_2 evolution exhibits a maximum followed by a spontaneous decrease of the O_2 level even though the actinic light is continuously applied. This is due to supersaturation of O_2. The nucleation of O_2 bubbles occurred at

certain stage of the reaction, eventually leading to the decrease of the O_2 level in the reaction mixture. The succeeding ON-OFF cycles give a response about 60 % of the one observed in the preceding cycle, indicating that partial inactivation of PS II occurs during the exposure of saturated actinic light. With a reduced incorporated amount of PS II (2μg Chl*a*/ml), the oxygen evolution became significantly smaller as expected (a lower trace).

Figure 4b compares the O_2-evolving activities of PS II/$Mal_3(Phyt)_2$ and PS II/EPC vesicles reconstituted by the same procedures. It is noted that the O_2-evolving activity is much higher for the PS II/$Mal_3(Phyt)_2$ than that of PS II/EPC, PS II/EPC gave only marginally measurable light-induced O_2 evolution.

(a)



(b)

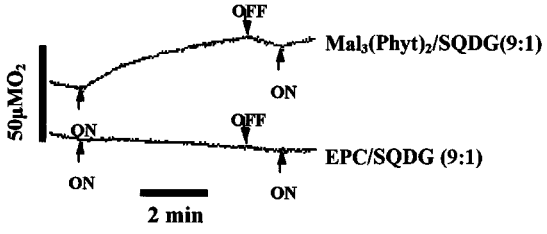


Figure 4. Light-induced oxygen evolution of reconstituted PS II at 40°C.
(a) Effect of incorporated amount of PS II in $Mal_3(Phyt)_2$/SQDG (9:1, mol/mol) vesicles. 10μgChl*a*/ml,[$Mal_3(Phyt)_2$] = 0.5 mM (upper trace); 2μg Chl*a*/ml, [$Mal_3(Phyt)_2$] = 0.5 mM (lower trace). The dashed arrows indicate the addition of 100 mM DCBQ (8μl each). The arrows labeled by ON and OFF ; saturated red light (λ> 610 nm) was applied and removed, respectively.
(b) Lipid effects. $Mal_3(Phyt)_2$/SQDG (9:1, mol/mol) vesicle and EPC/SQDG (9:1, mol/mol) vesicles. [Lipid] = 0.5 mM, [PS II] = 2μg Chl*a*/ml, [DQBQ] = 0.4 mM.

Incorporated amount of PS II and freeze-fracture electron microscopy images of PS II reconstituted vesicles.

Figure 5 shows freeze-fracture electron microscopy (FFEM) images of PS II reconstituted in $Mal_3(Phyt)_2$ vesicles (A, B) or EPC vesicles (C, D), where small particles were clearly observed in vesicle membranes. No such particles were observed for the pure $Mal_3(Phyt)_2$ or EPC vesicles. Dimensions of the small particles (14 nm×11 nm) are in fair agreement with the reported size of the monomeric PS II of *S. elongatus* , 15.5×10.5×6.5 nm[37,38], indicating that the small particles observed in the $Mal_3(Phyt)_2$ and EPC vesicles are most probably monomeric PS II. In addition, two fracture faces of the bilayer membranes, a convex surface (a hydrophobic face of an inner leaflet, A,C) and a concave surface (a hydrophobic face of an outer leaflet, B,D), both exhibited intramembraneous particles of a similar size, indicating PS II was incorporated into vesicles in a transmembrane manner. There is no clear difference between FFEM images of PS II/EPC and PS II/$Mal_3(Phyt)_2$ systems. Moreover, the incorporated amount of PS II estimated by an ultracentrifugation technique was practically the same for $Mal_3(Phyt)_2$ and various PC vesicles[36].

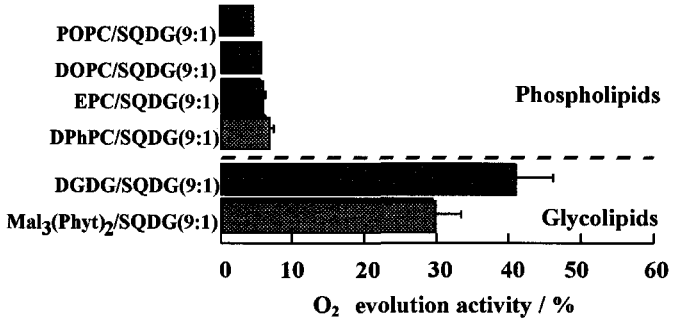


Figure 5. Lipid effects on the oxygen-evolving activity of PS II incorporated into vesicle at 40°C.
100% activity of oxygen-evolving activity was defined as the one measured in the Mes-Hepes/betaine buffer just before initiating reconstitution procedure, 1900 ± 50 μmol O_2/mg Chl a·h.
Significantly highr oxygen-evolving activity of PS II incorporated into the glycolipids, $Mal_3(Phyt)_2$ and DGDG, as compared with conventional PCs is seen.

Lipid effects on the O_2-evolution activity of the reconstituted PS II.

Figure 6 compares the O_2 evolution activity of the PS II/$Mal_3(Phyt)_2$ system with that of a range of PS II/ PC vesicles, where all the PS II reconstitution into PC vesicles was performed by the same procedures as that into $Mal_3(Phyt)_2$ vesicles. A 100 % of O_2 evolution activity was defined as the one measured for PS II in the Mes/Hepes/betaine buffer just before initiating reconstitution procedures. It is interesting that the glycolipids, $Mal_3(Phyt)_2$ and DGDG exhibit significantly better performance than PCs examined, i.e., 30~40 % of the original activity for the glycolipids, while only less than 5 % for all PCs examined. PS II reconstituted in $Mal_3(Phyt)_2$ vesicles displays 5~6-fold higher activity than

that in the PC vesicles.

As the activity of PS II/DPhPC system is comparable to those of the various PS II/EPC systems, the phytanyl chain is not a major factor to retain the activity of the PS II/$Mal_3(Phyt)_2$ system. The results strongly suggest some crucial effects of the sugar headgroups in supporting the PS II activity.

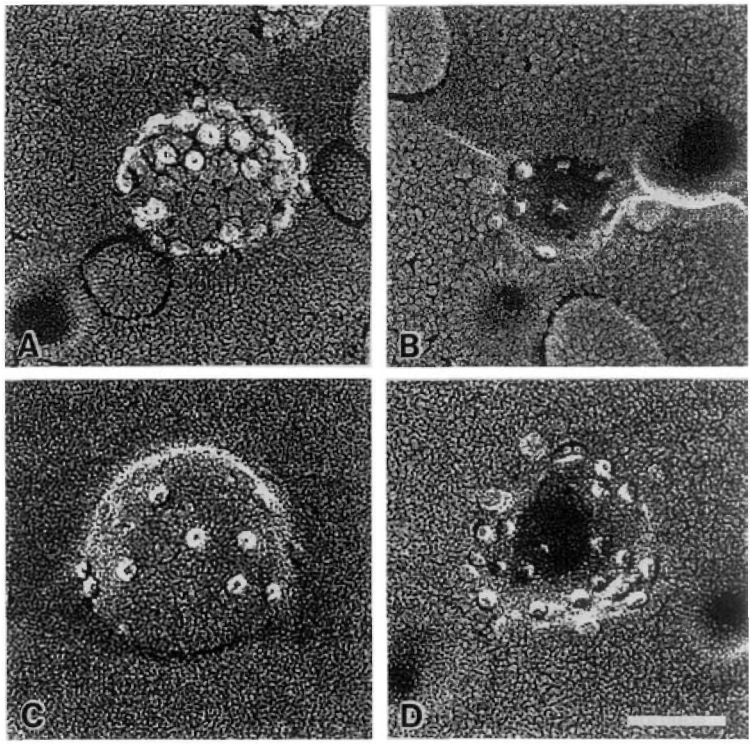


Figure 6. FFEM images of PS II reconstituted vesicles.
(A) Fractured convex half and (B) concave half of $Mal_3(Phyt)_2$/SQDG (9:1, mol/mol) vesicle; (C) convex half and (D) concave half of EPC/SQDG (9:1, mol/mol) vesicle.
Bar = 50 nm. Samples were prepared at the initial concentrations of 2 mM lipid, 0.22 mM SQDG and 8μg Chl*a*/ml PS II and concentrated by ultracentrifugation in the presence of Mes-Hepes/betaine buffer (pH 7.0).

DISCUSSION

Membrane lipid composition -hydrophobic matching and interfacial charges-

As the length of the hydrophobic chain determines the thickness of the hydrophobic part of the lipid bilayer, this should correspond closely to the dimension of the native membrane. Thylakoid membranes of *S. elongatus* grown at 55°C are composed of four major glycerolipids: 56 mol % monogalactosyldiacylglycerol (MGDG), 23 mol % digalactosyldiacylglycerol (DGDG), 15 mol % SQDG, and 6 mol % phosphatidylglycerol (PG)[36]. As to the hydrocarbon chain composition, 74 mol % of the hydrophobic chains are C_{16} chains: 56 mol % of saturated palmitoic acid ($C_{16:0}$) and 18 % of monounsaturated palmitoleic acid ($C_{16:1}$)[36]. Thus, the use of the phytanyl-chained glycolipid that has 16 carbon atoms in the main chain, appears reasonable in terms of the hydrophobic chain length. As SQDG is the major anionic lipid constituent of the thylakoid membrane of*S. elongatus* and would be essential to the PS II activity[39], we employ a mixture of $Mal_3(Phyt)_2$/SQDG (9:1 mol /mol) as a reconstitution matrix. As discussed in the following section, the presence of charged SQDG in $Mal_3(Phyt)_2$ vesicles is also crucial to obtain the stable vesicle suspension in the Mes-Hepes/betaine buffer. In the absence of SQDG, $Mal_3(Phyt)_2$ vesicles aggregate to form precipitates in the buffer[33].

Why the salt-induced aggregation of the non-ionic $Mal_3(Phyt)_2$ vesicles occurrs ?

As $Mal_3(Phyt)_2$ is a non-ionic compound, the colloidal instability of the $Mal_3(Phyt)_2$ vesicles in the buffer is rather surprising and unexpected. This, however, can be interpreted in terms of a common potential profile between non-ionic "glycolipid surfaces" in water. A typical potential profile is shown in Figure 7 where the interaction free energy between the $Mal_3(Phyt)_2$ monolayers estimated from the force measurements is shown[40]. The inter-glycolipid membrane potentials have also been measured for several synthetic glycolipids such as OG[41,42], N-lauroyl lactitol[43], and naturally occurring glycolipids[44]. Interestingly, despite the diverse headgroups and the lipid molecular structures examined, all the measured potential profiles have one feature in common, that is to say "hard-surfaces" interacting via a short range attractive potential such as shown in Figure 7. The potential minima are significantly larger than those for PC. The "hydration forces", which are often believed to be major forces to stabilize glycolipid vesicles, appear very short-ranged and not effective for the surfaces to repel each other. In neutral aqueous solutions (pH 5.5 - 7), $Mal_3(Phyt)_2$ vesicles are stabilized largely by weak double layer forces with the surface potentials of about -10 mV. The interfacial charges arise from the "adsorption" of OH^- at the vesicle-water interface[33]. Only a small increase in salt concentration is, therefore, sufficient to make the energy barrier comparable to or below kT, eventually leading to the vesicle aggregation. The surface charges of SQDG generate long-range repulsive double layer forces to stabilize the vesicles.

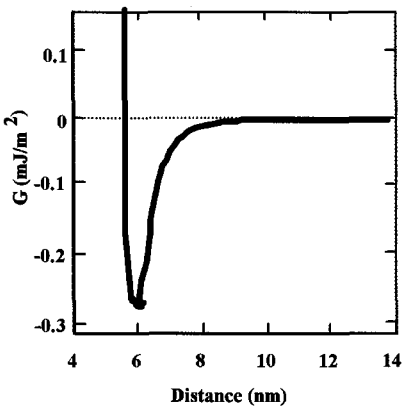


Figure 7. The schematic drawing of the interaction free energy per unit area, G, for two parallel $Mal_3(Phyt)_2$ layers in a neutral to acidic pH regime [redrawn from the force curves in a reference 40]. The distance D is defined as $D = 2l + d$, where $2l$ and d represent the thickness of two $Mal_3(Phyt)_2$ monolayers and the intervening water layer thickness, respectively.

Possible protective effects of glycolipids against destabilization of PS II.

Although the O_2 evolution activities of the PS II/$Mal_3(Phyt)_2$ or the PS II/DGDG systems are distinctly higher than those of the PS II/ PC systems, the activities were only 30 ~ 40% of the original PS II activity (Fig. 4). We noted that a Bio-Beads method[45], an alternative method for removing surfactants more rapidly than dialysis, significantly enhances the activity of the reconstituted PS II. In the Bio-Beads method, the PSII/lipid solubilized solution was treated with Bio-Beads SM-2 at 25°C for 3 h to obtain PS II/lipid vesicles. The PS II/$Mal_3(Phyt)_2$ system thus prepared displayed more than 80 % of the original O_2 evolution activity, while the PS II/EPC system now exhibited about 30 % of the original activity. This strongly suggests that the destabilization of PS II was largely due to the exposure of PS II to high levels of OG. The glycolipids, again in the Bio-Beads method, support significantly higher activity of the reconstituted PS II. The glycolipids appear to protect PS II from the OG-induced destabilization.

CONCLUDING REMARKS

Though the experimental parameters and types of membrane proteins examined here were not yet extensive, the results reported in this (and a following report) demonstrate the potential of synthetic glycolipid lyotropic LCs. The synthetic glycolipids that are "biocompatibile" and display low values of T_m (< 0 °C) will open up a new route to construct novel biomolecular archtectures involving membrane proteins.

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Table 1. comparison of $Mal_3(Phyt)_2$ and egg york phosphatidyl choline EPC

EPC $Mal_3(Phyt)_2$
Phaselamellar (L_α) lamellar (L_α)$T_m < 0$ °C< 0 °C
Chemical stability at 25° C < 1 ~ 2 week≥ 1 year
Permeability (H^+/OH^-)
PSII activity < 5 % ~ 35 % (dialysis)~ 30 % ≥ 80 % (biobeads)

Each lipid/water system displays a specific temperature, T_m, (gel-liquid cryataline phase transtransition temperature[10]) above which a hydrated solid surfactant transforms into a variety of liquid crystalline phases. These fluid phases are crucial to the functions of surfactant/water systems[11]. At temperatures below T_K, on the other hand, a surfactant precipitates as a hydrated solid and is difficult to use. Thus, to be usable as a surfactant, T_K must be lower than working temperatures, most preferably well below room temperature[12]. As demonstrated in this communication, the phytanyl group is large enough to form the inverted phases and at the same time effective in keeping the T_K values well below room temperature. Though other branched chains such as mono-, dimethyl iso- and methyl- and ethyl-antesio branches, consistently depress T_K[43], their effectiveness in depressing T_K is far less than that of the phytanyl group. Unsaturated alkyl chains do not appear to be as

efficient in depressing T_K of sugar-based surfactants[44].

MOLECULAR REQUIREMENTS

Let us first consider the lipid molecular structures required. First is the hydrophobic matching. The length of the hydrophobic chain determines the thickness of the hydrophobic part of the lipid bilayer, this should correspond closely to the dimension of the native membrane. As most biological membranes contain diacylglycerol lipids with hydrophobic chain lengths of 16 ~ 18 carbon atoms. Thus, synthetic lipids should possess relatively long hydrocarbon chain length, e.g., 16 ~ 18 carbon atoms.

Proteins are fucntional only in the fluid membrane conditions, T_m of the lipids should be low, most preferebly below 0°C. First of all, the lipids should not inhibit protein functions and induce denaturation. For this we choose sugars as their headgroups, since sugars are empirically known to stabilize protein functions and structures in aqueous solution[29]. Moreover, rich variety of the carbohydrate chemistry can provide powerful means to control their aqueous phase structures.

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ARTIFICIAL PHYTANYL-CHAINED GLYCOLIPID VESICLE MEMBRANES WITH LOW PROTON PERMEABILITY ARE SUITABLE FOR PROTON PUMP RECONSTITUTION MATRICES

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ABSTRACT

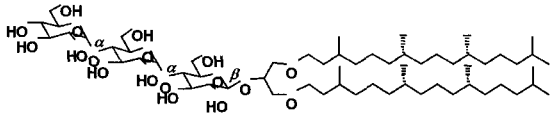
A light-driven proton pump, bacteriorhodopsin (BR) has been reconstituted into an artificial phytanyl-chained glycolipid, 1,3-di-*o*-phytanyl-2-*o*-(β-D-maltotriosyl)glycerol ($Mal_3(Phyt)_2$) vesicle membranes, of which proton permeability is much lower than those of straight-chained phospholipid, egg yolk phosphatidylcholine (EPC) membranes. Although BR had an inside-out orientation and translocated protons from the vesicle outside to the inside during illumination in both cases of $Mal_3(Phyt)_2$ and EPC membranes, BR reconstituted into $Mal_3(Phyt)_2$ membranes exhibited a prolonged transmembrane pH gradient due to those lower proton leak rates. This observation suggests the artificial phytanyl-chained glycolipid membranes with low proton permeability are advantageous for the stable and functional reconstitution of energy-conversion membrane proteins, allowing an efficient generation and/or consumption of a transmembrane proton motive force.

INTRODUCTION

The utilization of lipid membranes, mainly phospholipid membranes, has been extensively examined to develop liposomal drug delivery systems as well as biotechnological devices such as biosensors, bioreactors, etc., as structural elements of such devices [1-4]. On the other hand, glycolipids have been employed not as structural elements but as functional ones [5]. In general, however, the problems of conventional lipids for these applications are their chemical, physical, and biological instability. To overcome these problems, several methods for stabilization of lipids have been developed and one of the methods is to utilize lipids from archaea, which recently been identified as the third biological kingdom and inhabit under extreme conditions such as very high temperature, low or high pH, or high salt concentrations [6, 7]. Their lipid membranes are therefore considered to be

chemically and physically stable, and have been shown to be stable with low permeabilities to proton and other ionic or nonionic solutes [6-15]. Because the barrier function of membranes is critical for the functioning of cell and intracellular organelles, where a proton motive force is established [7], archaeal membranes are expected advantageous for avoiding short-circuiting of the pH gradient, and therefore promising as a element of nano-devices containing functional proteins which generate and/or consume the proton motive force such as a bio-hydrogen generation system.

In this paper, we will describe one of examples, where artificial archaeal glycolipids are applied to the construction of nano-devices containing energy-conversion membrane proteins, by employing the phytanyl-chained glycolipid we have recently developed, i.e., 1,3-di-*o*-phytanyl-2-*o*- (β-D-maltotriosyl) glycerol ($Mal_3(Phyt)_2$, Fig. 1) [16,17] and natural proton pump, bacteriorhodopsin (BR) derived from purple membranes of the extremely halophilic archaeon *Halobacterium salinarium* S9 [18].



$Mal_3(Phyt)_2$

Figure 1. Chemical structure of the artificial phytanyl-chained glycolipid, 1,3-di-*o*-phytanyl-2-*o*- (β-D-maltotriosyl) glycerol ($Mal_3(Phyt)_2$).

MATERIALS AND METHODS

Materials

$Mal_3(Phyt)_2$ was prepared as described previously [16]. EPC and a sulfoglycolipid sulfoquinovosyldiacylglycerol (SQDG) were purchased from Sigma Chemical Co. (St. Louis, MO) and Lipid Products (South Nutfield, Surrey, UK), respectively. n-Octyl-β-D-glucoside (OG) was from Dojindo (Kumamoto, Japan). Bio-Beads SM-2 was from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of a guaranteed reagent grade and purchased from Wako Pure Chemical Industries (Osaka). Bacteriorhodopsin (BR) was prepared from purple membranes of the extremely halophilic archaeon *H. salinarium* S9 [19]. Purple membranes were solubilized with 40 mM OG in 25 mM phosphate (NaH_2PO_4-Na_2HPO_4) buffer solution (pH 6.9) at room temperature in the dark [20], and used without separation of endogenous lipids.

Incorporation of BR into vesicle membranes

A mixture of lipid and SQDG (9 : 1 mol/mol) was solubilized with OG in 75 mM K_2SO_4 unbuffered solution (pH 6-7). OG-solubilized BR was then added to the solubilized lipid to give a final BR concentration of 100 μg/ml of 75 mM K_2SO_4 unbuffered solution. The solubilized lipid/SQDG/OG/BR mixture was dialyzed against 100-fold volume of 75 mM K_2SO_4 unbuffered solution containing Bio-Beads SM-2 for 18 h at 4°C in the dark. During the dialysis, the dialysis bag was continuously rotated using a rotary mixer to prevent the

precipitation of glycolipid aggregate [21]. Average diameters of the BR-reconstituted vesicles were measured with an Otsuka ELS-800TS electrophoretic light scattering apparatus using dynamic light scattering mode. Both samples were polydisperse, and the diameters were 240 ± 140 nm and 220 ± 120 nm for $Mal_3(Phyt)_2$ and EPC vesicles, respectively.

Assay of proton pumping activity of BR

The light-driven proton pumping activity of the reconstituted BR was measured with a GS-5015C combined glass electrode connected to an HM-14P pH meter (Toa Electronics, Tokyo) at 25°C. To abolish the diffusion potential across the membrane during the reaction, 5 μM valinomycin (molar ratio of valinomycin to total lipid $\sim 3\times10^{-3}$) was added into the suspension prior to measurements. The actinic beam for the excitation of BR was introduced into a thermostated glass cell with an optical fiber from a 150 W Xe arc lamp (Kenko, Tokyo) through a Toshiba Y50 glass filter ($\lambda > 500$ nm). The light intensity was set at the maximum output of the lamp. The pH changes were calibrated by the addition of small amount of HCl. The concentration of BR was determined by the modified Lowry method [22] using bovine serum albumin as a standard.

Freeze-fracture electron microscopy (FFEM)

The samples of BR-reconstituted vesicle (100 μg BR/1.5 mM lipid) were quick-frozen using the technique of Heuser [23], and fractured in a Balzers BAF 400D freeze-fracture apparatus (Balzers, Liechtenstein). The replicas were obtained by rotary shadowing with platinum/carbon of ca. 7 nm thick and carbon of ca. 25 nm, and then examined in a Philips CM200 Ultra Twin electron microscope at 200 kV.

RESULTS

FFEM observations of BR-reconstituted vesicle membranes

Figure 2 shows FFEM images of $Mal_3(Phyt)_2$/SQDG (9 : 1 mol/mol) vesicle membranes in the presence (Fig. 2A, B) or in the absence (Fig. 2C, D) of BR. Small particles were observed in Figure 2A, B, whereas no such particles were observed for pure $Mal_3(Phyt)_2$/SQDG vesicles. In addition, two fracture faces of the bilayer membranes, a convex surface (a hydrophobic face of an inner leaflet, Fig. 2A) and a concave surface (a hydrophobic face of an outer leaflet, Fig. 2B) exhibited intramembraneous particles, suggesting BR was incorporated into vesicles transmembraneously.

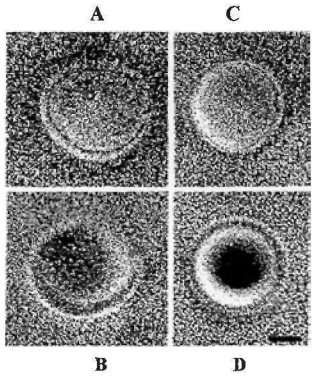


Figure 2. FFEM images of BR reconstituted and protein-free vesicles.
(A) Fractured convex half and (B) concave half of Mal_3 $(Phyt)_2$/SQDG (9 : 1 mol/ mol) vesicle containing BR; (C) convex half and (D) concave half of protein-free vesicle. Bar = 50 nm. Samples were prepared at the initial concentration of 2 mM Mal_3 $(Phyt)_2$, 0.22 mM SQDG and 50μg/ml BR and concentrated by ultracentrifugation in 75 mM K_2SO_4 buffer solution.

Functioning of BR-reconstituted vesicle membranes

In both cases of Mal_3 $(Phyt)_2$/SQDG and EPC/SQDG vesicle membranes, the reconstituted BR exhibited light-induced alkalinization of the external aqueous phase and relaxation in the dark as shown in Figure 3. This shows the reconstituted BR translocates protons during illumination, and the orientation of BR in both membranes is in the same direction, opposite to that found in native whole cells (inside-out orientation). The proton movement was assumed to obey the first-order kinetics: $\ln(1 - \tilde{\Delta}/\Delta_s) = -k_L t$, where Δ and Δ_s are the extent of proton uptake at time t of illumination and at steady-state, respectively, while k_L is the rate constant for proton translocation under illumination. The release of protons in the dark stage also follows the first-order kinetics: $\ln(\tilde{\Delta}/\Delta_s) = -k_D t$, where k_D is the rate constant for a light-independent decay. Although the initial proton pumping rates R_0 (= $k_D\Delta_s$) for Mal_3 $(Phyt)_2$ and EPC vesicles were comparable and ranged from 0.1 to 0.4 mol H^+/mol BR·s, there were marked differences in the decay rates in the dark as can be seen in Figure 3. Figure 4 shows the half-life of the decay in the dark $\tau_{1/2}$ (= $\ln 2/k_D$) as a function of lipid concentration for Mal_3 $(Phyt)_2$ or EPC vesicles. Obviously, Mal_3 $(Phyt)_2$ vesicles exhibit ca. 10-fold longer half-life than EPC vesicles, suggesting the proton efflux from the internal aqueous phase of Mal_3 $(Phyt)_2$ vesicles is much slower than that of EPC vesicles.

DISCUSSION

In general, membrane protein reconstitution experiments require the use of various kinds of buffer solution containing inorganic salts essential to the functioning of membrane proteins. However, as previously reported, the vesicle membranes consisting of Mal_3 $(Phyt)_2$ alone are much more susceptible to changes in pH and/or ionic strength compared to PC vesicle membranes [24]. The use of concentrated buffer solution may lead to lowering of the colloidal stability of Mal_3 $(Phyt)_2$ vesicles or make the vesicle preparation difficult.

Therefore, when $Mal_3(Phyt)_2$ vesicle membranes are applied to the reconstitution matrices for membrane proteins, any means to improve the colloidal stability of this glycolipid vesicles should be used, and one of the simplest ways is an addition of a small amount of charged lipids into the vesicle membranes, leading to the enhancement of repulsive double-layer force and the inhibition of the vesicle aggregation. As the negatively charged glycolipid SQDG is the major anionic glycolipid constituent of polar lipids (ca. 15 mol% of polar lipids) in thylakoid membranes, this uncommon glycolipid was selected and used for the purpose of improving the colloidal stability.

Native purple membranes of *H. salinarium* S9 consist of diphytanyl ether analog lipids: ca. 70 mol% phosphatidylglycerophosphate (PGP), ca. 20 mol% glycolipid sulfate (GLS), and ca. 7 mol% phosphatidylglycerosulfate (PGS), and a few mol% phosphatidylglycerol (PG) [25,26]. These lipids are all negatively charged as can be seen in Figure 5. The reconstitution of BR so far has been carried out into various lipid membranes [27-30]. In the case of vesicles consisting of native purple membrane lipids, it has been reported that only GLS bearing a triglycosyl moiety forms vesicle membranes with low ion permeability [28,31], and a mixture of PGP and GLS at a ratio close to that found in native purple membranes creates the best lipid environment for the proton-translocating activity of BR [29]. The chemical structure of GLS is close to that of $Mal_3(Phyt)_2$, and for that reason, the phytanyl-chained glycolipid is expected to be suitable for BR reconstitution.

BR is the well-known light-driven proton-translocating protein to generate a transmembrane proton motive force [18], and can be reconstituted efficiently into various lipid membranes [27-30]. By applying the OG-solubilization-dialysis method, BR was incorporated into vesicle membranes as indicated by FFEM images (Fig. 2) and proton pumping (Fig. 3). According to a number of reports [27-30], the preferred orientation of BR in the reconstituted vesicle membranes appears to be opposite to that in whole cells (inside-out orientation). This is also the case for $Mal_3(Phyt)_2$/SQDG vesicle membranes under the present conditions. Although the proton-translocating activity of BR in $Mal_3(Phyt)_2$ membranes was comparable to that in EPC ones under the present conditions, the decay of the pH gradient across $Mal_3(Phyt)_2$ membranes in the dark is much slower than that across EPC ones, mostly reflecting the difference in the proton permeability across each membrane as previously reported [32,33]. This result obviously indicates that $Mal_3(Phyt)_2$/SQDG vesicles are more suitable for BR reconstitution from the viewpoint of an efficient generation of the transmembrane proton motive force.

In conclusion, the potential of the artificial phytanyl-chained glycolipid, $Mal_3(Phyt)_2$ membranes as matrices for reconstitution of membrane proteins was demonstrated by employing BR. The structural characteristics of $Mal_3(Phyt)_2$ membranes with low proton permeability and bearing the saccharide moiety will be advantageous for the stable and functional reconstitution of various types of energy-conversion membrane proteins, allowing an efficient generation and/or consumption of the transmembrane proton motive force. We are now extending our research to reconstitute other type of proton pump, photosystem 1, which is a key element for bio-hydrogen production system, into the artificial phytanyl-chained glycolipid membranes.

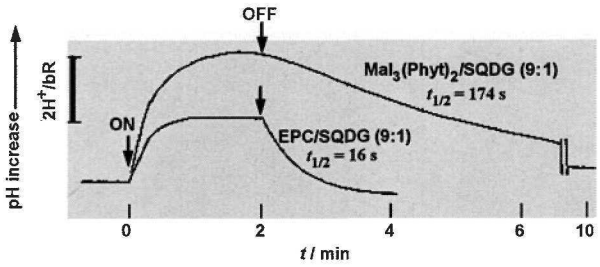


Figure 3. Light-induced proton-translocation of BR reconstituted into vesicle membranes at 25℃.
Upper trace: $Mal_3(Phyt)_2$/SQDG (9 : 1 mol/ mol); lower trace: EPC/SQDG (9 : 1 mol/mol). [lipid] = 1.5 mM; [SQDG] = 0.17 mM; [BR] = 100μg/ml. The medium was 75 mM K_2SO_4 unbuffered solution (pH 6-7).

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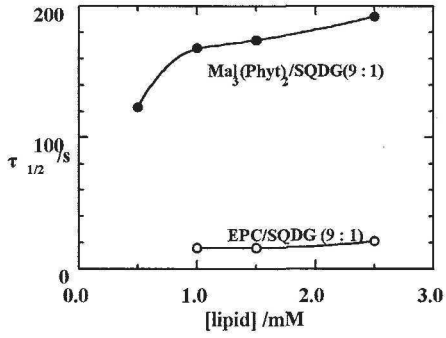

Figure 4. Half-life $\tau_{1/2}$of pH gradient decay in the dark as a function of lipid concentration for $Mal_3(Phyt)_2$ or EPC vesicles after light-induced proton translocation by BR at 25℃.
[BR] = 100μg/ml.
The medium was 75 mM K_2SO_4 unbuffered solution (pH 6-7).

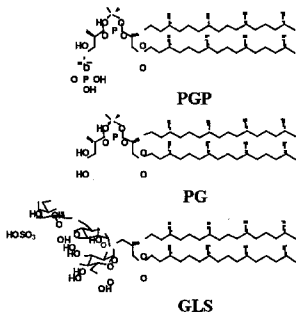

Figure 5. Chemical structures of main lipids of purple membranes from *Halobacterium salinarium* S9; phosphatidylglycerophosphate (PGP), phosphatidylglycerol (PG) and glycolipid sulfate (GLS). For GLS, the headgroup is a galactosyl-3-sulfate-β-D-1→ 6-mannosyl-α-D-1→2-glucosyl moiety. Adapted from refs. [25,26].

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AMPHIPOLS: STRATEGIES FOR AN IMPROVED PS2 ENVIRONMENT IN DETERGENT-FREE AQUEOUS SOLUTION

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ABSTRACT

Amphipols (APS) are amphipathic polymers designed to keep membrane proteins soluble in the absence of detergents. In the present study, we show that APS are able to keep PS2 core complexes from the thermophilic cyanobacterium *Thermosynechococcus elongatus* soluble in aqueous solutions free of detergent micelles. Upon size-exclusion chromatography, PS2/AP complexes appear mainly comprised of dimeric reaction centers, with a slightly larger Stokes radius than PS2/dodecylmaltoside complexes. Their oxygen-evolving activity is slightly higher than that of samples kept in detergent solution. Upon storage in the dark at room temperature, the biochemical stability of the centers is remarkably high and similar in both environments, with more than 50 % of the original activity being retained after one week of incubation at room temperature in the dark.

KEYWORDS:

Amphipols, PS2, long-term stability

INTRODUCTION

Transmembrane proteins are adapted to an environment comprised of two distinct aqueous media and the highly complex membrane phase [1]. Handling them in aqueous solution requires their complexation by amphipathic molecules that screen their hydrophobic transmembrane surface from contact with water. Traditionally, this role is fulfilled by detergents. Detergents are small surfactants that cooperatively assemble at the transmembrane surface of the protein at concentrations close to their critical micellar

concentration (CMC) [2]. When the concentration of free detergent is lowered below the CMC, most detergent molecules desorb and the protein becomes insoluble. Membrane proteins therefore have to be handled in the presence of excess detergent micelles. Detergents being, by definition, endowed with dissociating properties, this frequently proves harmful: perturbation of functional properties if not downright inactivation by detergents is a recurrent concern in membrane protein biochemistry [see *e.g.* refs.3, 4].

'Amphipols' (APS) are amphipathic polymers designed for making membrane proteins hydrophilic [5; for a review, see ref. 6]. When added to a membrane protein in detergent solution, they form with the detergent a mixed layer around the transmembrane surface of the protein [7]. Upon detergent removal, they stick to the protein, keeping it water-soluble [5; Fig. 1]. Membrane proteins are not denatured by APS and, as a rule, tend to be more stable as AP-trapped complexes than they are in detergent solutions. The functionality of AP-complexed proteins has received relatively little attention yet. Reversible inhibition by pure APS has been reported in the case of the sarcoplasmic reticulum calcium pump [8], while the nicotinic acetylcholine receptor, whose allosteric transitions are altered in detergent solution, recovered native-like properties after supplementation with APS and dilution under the CMC of the detergent[9]. Observations on these and other proteins indicate that the stability and functionality of AP-trapped membrane proteins can be modulated by such factors as the presence of lipids and/or residual detergent and the charge density of the polymer [reviewed in ref.6; see below, *Discussion & Outlook*].

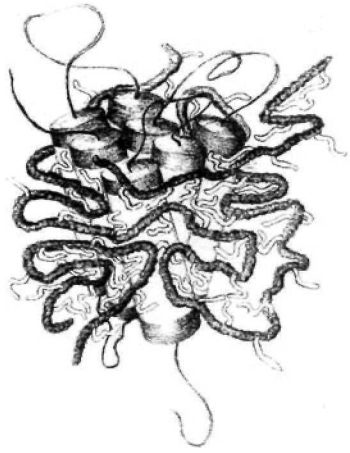

Figure 1. Sketch of a protein-amphipol complex [from ref. 5].

APS represent an attractive novel way of stabilizing membrane proteins under conditions where they can be exposed to detergent-free media. In the present work, we report some preliminary experiments aimed at complexing with APS a photosynthetic PS2 reaction center core complex, that from the thermophilic cyanobacterium *Thermosynechococcus elongatus*.

MATERIALS & METHODS

Amphipol synthesis

Amphipol A8-35 is a polyacrylate-based AP, with an average final molecular mass of ~ 8 kDa[5]. 25 % of the original carboxylic groups have been derivatized with octylamine, making the polymer amphipathic, 40 % are blocked with isopropylamine, in order to reduce the charge density along the chain, and 35% are left free, conferring solubility at neutral and basic pH (Fig. 2). A8-35 was synthesized by P. Hervé and F. Giusti (UMR 7099) as described in ref. 5.

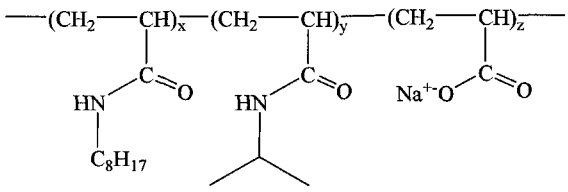

Figure 2. Molecular structure of a polyacrylate-based amphipol [see ref. 5].
For amphipol A8-35, x = 0.25, y = 0.40, z =0.35.
The distribution of the three types of units is random.

Isolation of PS2 core complexes

PS2 core complexes from *T. elongatus* were prepared by a two-step HPLC procedure as described in ref. 10, with the following modification: after solubilization of the thylakoid membranes with dodecyl-β-D-maltoside (β-DM) and centrifugation, the supernatant was loaded onto 27ml of 14 % sucrose solution, w/w, in buffer B (20mM MES pH 6.5, 10mM $CaCl_2$, 10mM $MgCl_2$, 0.5M mannitol and 0.03 %β-DM), layered on top of an 80% sucrose cushion in the same buffer. After 18h of centrifugation (4°C, 83.000 ×*g*), the core centers - visible as a green band in the upper layer - were collected with a syringe and prepared for the first chromatographic step.

Analytical size exclusion chromatography

Analytical size exclusion chromatography was performed on an Äkta Purifier (Pharmacia) using a 24-ml Superose 6 HR 10/30 column (Pharmacia). 250-µl samples in AP-buffer (20mM Tris buffer, pH 8.0, 100 mM NaCl), with or without 0.1 % β-DM, were injected onto the column equilibrated with the same buffer and eluted at a flow rate of 0.3 $ml \cdot min^{-1}$. Elution profiles were recorded at 220, 280 and 674 nm.

PS2 activity measurements

Rates of oxygen evolution were measured at 30°C in a thermostated Clark-type oxygen electrode (Hansatech) using the continuous, saturating light of a 250-W cold light source (Halolux). PS2 core complexes were suspended in 20 mM MES, pH 6.5, 30mM $CaCl_2$,

10mM $MgCl_2$, 1M betaine, at a concentration of 2-5 µg chlorophyll (Chl)·ml^{-1}. AP-free samples were supplemented with 0.03 % β-DM. 2,6-Dichloro-*p*-benzoquinone (2,6-DCBQ) and ferricyanide (at 1 mM each) were used as artificial electron acceptors. Long-term stability of the oxygen evolving activity upon storage was investigated using an oxygen sensor consisting of a polymer optical fiber whose polished tip is coated with a planar oxygen-sensitive foil (Presens, Regensburg, Germany). The measurement is based on dynamic luminescence quenching by molecular oxygen [11]

RESULTS

Interaction of amphipols with PS2 core centers

The optimal AP/protein ratio to be used for forming water-soluble complexes was determined by trapping detergent-solubilized PS2 core centers under various conditions. Polyacrylate-based APS should preferably be handled at or above pH7.5 [6]. The ~1 % β-DM solution (~20 mM), containing 30 mg protein per ml, was therefore diluted 10-fold with AP-buffer (20 mM Tris pH 8) in order to raise the pH. This solution was then supplemented with various amounts of A8-35. After 15-min incubation at room temperature, the samples were diluted 10-fold again with detergent-free AP-buffer and incubated 2h at room temperature with 10 g BioBeads (Biorad) per g protein to remove monomeric detergent. The samples were centrifuged for 10 min at 55.000 × *g* and the Chl concentration of the supernatants measured.

Under these conditions, about 5 g A8-35 per g PS2 are sufficient to keep all PS2 soluble (Tab. 1). This is in the same range as observed with most membrane proteins [see *e.g.* ref. 5]. Although not determined in the present experiments, it is very likely that only a fraction of AP binds to PS2 centres, as consistently observed in other systems [7; see ref. 6].

Table .1 Determination of the optimal amphipol to PS2 ratio needed to keep PS2 soluble

	control 1	protein/A8-35 ratio (w/w)			Control 2
	-AP, -β-DM	1:3	1:5	1:10	-AP+ β-DM
Chl (µg·$µl^{-1}$)	0	0.0468	0.0493	0.0497	0.0503
soluble PS2	0%	93%	98%	99%	100%

Size exclusion chromatography analysis of PS2/AP complexes

The dispersity of PS2/A8-35 complexes was examined by size exclusion chromatography. The elution profile was similar to that in β-DM, with a major peak corresponding to the PS2 dimer and a shoulder corresponding to the monomer (Fig. 3). As observed before [reviewed in ref. 6], trapping with AP, therefore, preserved the particle distribution observed in detergent solution. As reported for the sarcoplasmic calcium ATPase [8] and other proteins [reviewed in ref. 6], AP-trapped PS2 dimers and monomers migrated slightly faster and yielded slightly broader peaks than their detergent-solubilized counterparts. There was evidence neither for monomerisation nor for the formation of large aggregates.

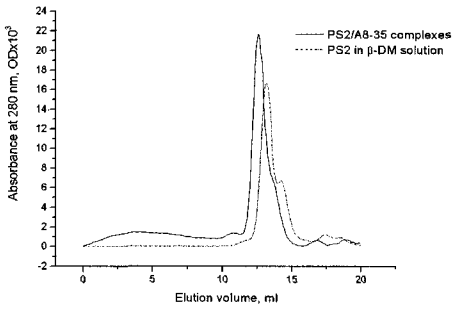

Figure 3. Size exclusion chromatography analysis of AP-trapped *vs.* detergent-solubilized PS2 core complexes.
75 µg AP-trapped PS2 were applied to a Superose 6 HR10/30 column (Pharmacia) equilibrated with detergent-free, AP-free buffer (20 mM Tris pH 8.0, 100 mM NaCl) and eluted at a flow rate of 0.3 ml·min^{-1} (*solid line*). For comparison,β-DM-solubilized PS2 (75 µg PS2 ≈ 12.5 µg Chl) was applied to the column under identical conditions, but with the buffer containing 0.1 % β-DM (*dotted line*). The experiments were run at 8°C.

Oxygen evolving activity of AP-trapped PS2 core centers

The water-splitting activity of detergent-solubilized and AP-trapped PS2 reaction centers was measured using a Clark electrode. As shown in Figure 4, differences between the two types of samples were minimal, with a slight decrease of activity when the pH of the β-DM solution was shifted to pH 8 and a moderate increase following addition of A8-35, dilution below the CMC of β-DM and treatment with BioBeads.

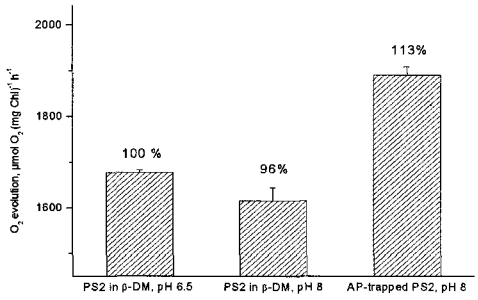

Figure 4. Oxygen evolving activity of AP-trapped *vs.* detergent-solubilized PS2 core complexes.
The oxygen evolving activity of β-DM-solubilized PS2 at pH 6.5 was used as 100 % control ("PS2 in β-DM, pH 6.5").
Before adding 5 g A8-35 per g PS2, the sample was diluted 10-fold with 20 mM Tris pH 8.0 ("PS2 inβ-DM, pH8").
Amphipol A8-35 was then added and the sample treated as described in § 5.1 ("AP-trapped PS2, pH8"). Oxygen evolution was measured with a Clark-type oxygen electrode under continuous, saturating light.

Long-term stability of PS2

Long-term stability is a prerequisite for many applications. The stability of *T. elongatus* PS2 centers was examined by measuring oxygen evolution after extended storage at room temperature, either in the dark or under continuous illumination with dim light. In the dark, both detergent-solubilized and AP-trapped PS2 were extremely stable, with ~50 % activity still present after over one week. There was no significant difference in stability between the two preparations (Fig. 5). In the light, on the other hand, the activity of both samples decreased rapidly, with the β-DM sample appearing slightly more stable. Repeating the measurements under anaerobic conditions in order to reduce the destructive effect of oxygen did not yield any improvement of long-term stability (data not shown).

DISCUSSION & OUTLOOK

Incorporating photosynthetic complexes in hydrogen production devices requires long-term stability at room temperature and immobilization on a solid support. The work presented here, while preliminary, represents a step in these directions. As to long-term stability, *T. elongatus* PS2 preparations, whether in β-DM solution or as complexes with APS, are much more stable at room temperature than comparable preparations from *Synechocystis* or spinach [M. Rögner *et al.*, unpublished results]. At room temperature in the dark, the stability of AP-trapped centers was comparable to that of β-DM solubilized centers; under dim light, AP-trapped centers appeared slightly less stable.

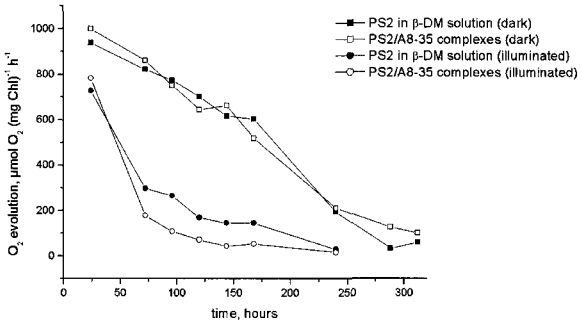

Figure 5. Long-term stability of oxygen evolving activity of AP-trapped *vs.* detergent-solubilized PS2 core complexes.
PS2 samples (AP-trapped,*open symbols*; in β-DM, *filled symbols*) in AP-Buffer (pH 8) were kept at room temperature in darkness (*squares*) or illuminated with continuous dim light (*circles*).
Oxygen evolution was determined by a FIBOX oxygen sensor (see *Materials & Methods*, § 4.4) under saturating light.

There have been few studies to date of the functionality and stability of AP-trapped photosynthetic reaction centers. *Rhodobacter sphaeroides* reaction centers were shown to remain intact following trapping with AP A8-75 (a more highly charged analog of A8-35), but neither their functionality nor their stability over time were studied[5]. *Synechocystis* PCC 6803 PS1 reaction centers trapped with A8-35 and deposited on a gold electrode have been shown to be electrochemically active, but their long-term stability has not been studied[12]. The photochemical activity of A8-35-trapped pea PS2 reaction centers, measured at room temperature by the accumulation of the pheophytin free radical upon illumination, was found to be intermediate between that in CHAPS and in β-DM solutions [A. Zehetner & H. Scheer, personal communication ; ref.13].

As mentioned above, one rationale for the design of APs was to alleviate the destabilizing effect of detergents. Usually -but not always-, AP-trapped proteins indeed appear to be stabilized as compared to their detergent-solubilized counterparts[5, 8]. The difference can be spectacular : in the case of calcium-free Ca^{2+}-ATPase, a particularly fragile protein, the enzyme solubilized in the presence of lipids has a half-life of ~1h after addition of A8-35 and dilution below the CMC of $C_{12}E_8$, in contrast to a half-life of ~1-2 min in detergent solution[8]. Cytochrome b_6f, on the other hand, is slightly less stable once complexed with either A8-35 or A8-75 than it is in a lipid/detergent mixture[5]. For both proteins, the formation of ternary complexes with lipids (and/or, in the case of the Ca^{2+}-ATPase, detergent) enhances stability[5,8]. The underlying mechanism is not known. The effect could be partly due to lipids binding to specific sites and partly to a better screening of the transmembrane surface from water[6]. Bacteriorhodopsin, at pH 8 and 4°C, was found to be much more stable after trapping with A8-35 or A8-75 than it is in octylthioglucoside solution, but it definitely prefers the first, least charged AP[5]. The biochemical stability of pea PS2 reaction centers supplemented with A8-35 in detergent solution (either CHAPS or β-DM) and then diluted under the CMC of

the detergent depends both on the temperature and the nature of the detergent. At 4°C, the stability is highest for CHAPS-solubilized or AP-trapped centers (independent of the nature of the detergent solution they were trapped from) and lowest for β-DM. With the former three, there is practically no change in activity over 15 h. At 20°C, all samples are considerably less stable, and the stability order changes to CHAPS > {A8-35 from CHAPS} > {A8-35 from β-DM} > β-DM (A. Zehetner & H. Scheer, personal communication ; ref. 13). The latter observations confirm that, even below their CMC, detergents associate with the amphipol layer, which can affect the properties of the trapped protein.

Several approaches can be used to try and improve the long-term stability of AP-trapped *T. elongatus* PS2 preparations. Adding back some detergent is not convenient if the preparations are to be exposed to surfactant-free aqueous solutions, but forming ternary PS2/AP/lipid complexes is a promising direction to explore. The chemistry of APS also offers many resources. First, polyacrylate-based APS form a large family, with many possible variations as regards for instance the size of the polymers, their charge density, and/or the density and nature of the alkyl chains [see refs.5, 6]. Second, and perhaps more promisingly, work is in progress to develop APS that would remain soluble over a larger range of pH, such as non-ionic, zwitterionic or sulfonated APS [reviewed in ref. 6]. These would make it possible to handle AP-trapped PS2 reaction centers at acidic pH, where they are most stable, rather than at pH 8 as was done in the present experiments in order to optimize the solubility and monodispersity of the PS2/AP particles [*cf.* ref. 6]. We have shown previously that non-ionic de APS rived from Tris(hydroxymethyl)-acrylamidomethane can be used to stabilize membrane proteins in aqueous solutions in much the same manner as polyacrylate-derived APs do [14], which bodes well for the future of this approach.

With regards to immobilization, it has already been shown that AP-trapped proteins can be adsorbed onto solid supports, *e.g.* on immobilized metal columns, *via* a polyhistidine tag [ref. 15 and M.Z. & J.-L.P., unpublished data], on glutathione columns, after grafting of a GST moiety [H.A. Shuman & M.Z., unpublished data], or on the surface of a BIAcore chip, by avidin/biotin interaction[16]. Immobilization of AP-rapped *T. elongatus* PS2 reaction centers onto electrodes may conceivably be achieved by similar approaches, *e.g. i) via* a polyhistidine tag grafted onto one of the PS2 subunits, or *ii)* by chemical modification of the AP, engineered to permit a covalent or non-covalent reaction with the support. Immobilization by itself may provide a route towards bringing PS2/A8-35 complexes to an optimal, slightly acidic pH without inducing aggregation.

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MONOLAYERS AND LONGMUIR-BLODGETT FILMS OF PHOTOSYSTEM I ON VARIOUS SUBPHASE SURFACES

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ABSTRACT

Monolayers of a pigment-protein complex, photosystem I (PS I), were investigated on the NaCl, $CaCl_2$, and two kinds of cationic polyelectrolyte subphase surfaces. The monolayers were transferred onto hydrophobic substrate surfaces by a horizontal lifting technique. Both the *in situ* UV-vis spectra at the air-water interface and the spectra of the transferred Langmuir-Blodgett (LB) films were similar to the spectrum of PS I in buffer solution. Cyclic voltammogram measurement of a PS I/poly(viologen) complex LB film modified electrode showed well reversible redox waves of viologens, which indicated that the film may be useful for the development of a biounit to mimic the photoinduced electron transfer process in the photosynthetic system.

KEYWORDS:

Photosystem I, Monolayers, Langmuir-Blodgett films, Absorbance, Electrochemistry.

INTRODUCTION

Photosystem I is a membrane pigment-protein complex in green plants, algae as well as cyanobacteria, and undergoes redox reactions by using the electrons transferred from photosystem II (PS II) [1]. These membrane proteins are considered to be especially interesting in the study of monomolecular assemblies, because their structure contains hydrophilic area that can interact with the subphase as well as hydrophobic domains that can interact either with each other or with detergent and lipids [2]. Moreover, studies with such proteins directly at the air-water interface are expected to be a valuable approach for their two-dimensional crystallization.

Several research groups have reported monolayers and LB films of PS II [3-5], but to our knowledge, no research has been up to now done on the monolayers of PS I. As PS I under radiation can act as a mediator for the electrons transferring from electron donors to electron

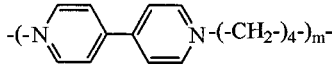
carrier, such as ferredoxin, or artificial electron carrier, such as methyl viologen, a biounit composed of PS I and viologen or ferredoxin may be interest in the development of a photoinduced electron transfer from electron donor to viologen and then to electron acceptor, the process of which can mimic the natural photosynthetic process and develop PS I based molecular devices [6]. In the present work, we report the monolayer behaviors and *in situ* absorbance of PS I on various subphase surfaces. These monolayers were transferred onto hydrophobic substrate surfaces and characterized by the UV-vis spectra, quartz crystal microbalance (QCM), and electrochemical analyzer.

EXPERIMENTAL SECTION

Materials

Poly(diallydimethylammonium chloride) (PDDA) was purchased from Aldrich Chemical Co. 2-Morpholinoethanesulfonic acid (MES) was from Wako Pure Chemical Industries Ltd. Poly(butylviologen) (PBV), shown in Scheme 1, was synthesized according to literature method and checked by HNMR [7]. Ultrapure water (18.3 MΩ) was prepared with a Milli-Q filtration unit of Millipore Corp.

Growth conditions of *Synechocystis* sp. PCC 6803 and subsequent isolation of thylakoid membranes were described in literature [8]. From these membranes, PS I was extracted by dodecyl-ß-D-maltoside treatment and purified by two successive HPLC steps, using first an anion exchange column (Poros 50 HQ, PerSeptive) and second a hydrophobic interaction column (Poros 20 Butyl, PerSeptive). The purification of trimeric PS I is described in detail in literature [9].



Scheme 1. Molecular structure of PBV used.

Monolayers and Langmuir-Blodgett films

The surface pressure-area (π-A) isotherm measurements and LB film transfer were performed with the use of a KSV 5000 minitrough (KSV Instrument Co., Finland) operated at a continuous speed for two barriers of 10 cm^2/min at 20°C. The buffer used in the present work was composed of 10 mM MES, 2 mM ascorbic acid sodium salt, and a given concentration of salt or polymers (pH =7.0). The accuracy of the surface pressure measurement was 0.01 mN/m. Monolayers of the PS I were transferred at 10 mN/m on hydrophobic substrate surface by horizontal lifting method.

Durability of the transferred PS I/PBV LB film in buffer solution was estimated by measuring its absorption spectra after the coated substrate was immersed in the MES buffer for a given time.

QCM Measurements

QCM measurements were carried out at air using AT-cut gold-coated quartz crystals with a resonant frequency of 9 MHz (5 mm-diameter, Seiko EG&G, Seiko Instruments Inc). Prior to use, the quartz crystals were cleaned with water and ethanol. The frequency was measured before and after the monolayer transfer with the use of a Seiko EG&G model 917 quartz crystal analyzer.

Spectroscopic measurements

In situ absorption spectra at the air-subphase interface were measured using two optical fibre waveguides connected to the light source and photodetector (MCPD-100 PHOTAL Otsuka Electronics multichannel spectrometer, Japan). The light beam passed through the monolayer, reflecting the light beam via a concave mirror placed just below the water surface. The UV-vis spectra of the transferred LB films were measured using a Shimadzu UV-1601 UV-vis spectrophotometer (Japan).

Electrochemical Measurements

Cyclic voltammetric (CV) experiments were done by using a BAS 100B electrochemical analyzer (USA). A Pt wire and Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively, and an ITO substrate, coated with one layer of PS I/PBV LB film, was used as the working electrode with 10 mmol/L KCl as the electrolyte. An initial potential of -0.20 V was applied for 2 s, and subsequently cyclic scans to a final potential of -1.30 V were done for 10 cycles. All electrochemical measurements were done under an Ar atmosphere at room temperature.

RESULTS AND DISCUSSION

Monolayer behaviors of PS1 on various subphase surfaces

It has been reported that cationic ions, such as Na^+ and Cd^{2+}, in the subphases are important for the formation of stable, active monolayers of PS II CC or bacterial reaction center from *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* [2, 10]. We previously found that $CaCl_2$, poly-L-lysine and PBV in the subphase are also able to stabilize the monolayer formation of a hydrogenase from the phototropic bacterium *T. roseopersicina* [11,12]. Based on the reported results, here, we investigated the PS I monolayer behaviors in four kinds of subphase surfaces. Figure 1 shows π-A isotherms for the monolayers of PS I on the (0.1~1) M NaCl, 0.02 M $CaCl_2$, 1 mg/mL PDDA, and 0.1 mM PBV subphase surfaces at room temperature. The curves indicate an increase of the surface pressure during the compression, which suggests that PS I monolayers are formed on those subphase surfaces. Although no significant difference was recorded for these curves, the following features are still much important. First, the surface pressure of PS I monolayer increases with the increase of the NaCl concentrations in the subphase (Fig. 1a), which may be attributed to an interaction (probably adsorption) between PS I and Na^+ [2, 10]. Second, the surface pressure increases with more PS I spread on the $CaCl_2$ subphase surface, but no significant difference is observed for the surface pressure after the monolayer is completely compressed. This means the monolayer properties are dominated by the PS I unit, though the cationic ions show some effects at lower surface pressures. Third, the surface pressure after PS I spread on the 0.02 M $CaCl_2$ subphase surface is even higher than that on the 0.1 M NaCl subphase surface, indicating a stronger interaction between PS I and Ca^{2+} than that between PS I and Na^+.

Although no PS I monolayers were studies up to now, some research groups have reported the monolayer properties of PS II at the air/nitrogen-water interface. Since the protein contains excess detergent molecules, it is difficult to calculate the area per PS I. Gallant et al. supposed that excess detergent should have left the interface at 33 mN/m, since the π-A isotherms of PS II monolayers all merge at this surface pressure [2]. In our experiments, although not all curves merge one surface pressure, the π-A isotherms show a phase transition point at around 25 to 30 mN/m.

No significant decrease was recorded from the area-time isotherm of the PS I monolayers at 10 mN/m, which indicates that the PS I monolayers formed are rather stable. This stability makes it possible for a multilayer deposition by the LB method.

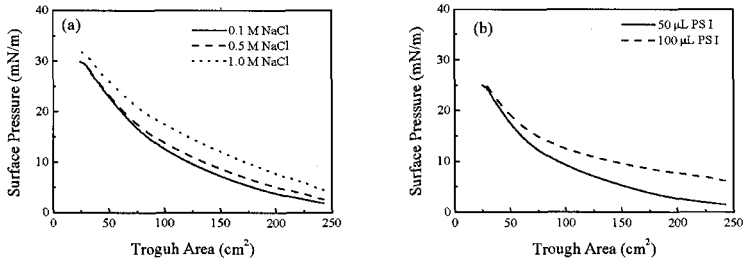


Figure 1. π-A isotherms for the PS I monolayers on (a) NaCl, (b) $CaCl_2$, (c) PDDA, and (d) PBV subphase surfaces.

In situ absorption spectra at the air-water interface

The absorption characteristics of PS I were measured on the four kinds of subphase surfaces during compression. As an example, Figure 2 shows the absorption spectra of the PS I monolayers on the PBV subphase surface under different surface pressures. Two absorption bands at about 420~450 and 676 nm increase with the compression, indicating the accumulation of the PS I to form a condensed monolayer. Compared to the absorption spectrum of PS I in solution (Fig. 3), the band at around 436 nm splits into two peaks. The wave-shaped small "band" between 470 and 630 nm is due to a low single-to-noise ratio on the water surface. These spectral features together with the π-A isotherms indicate that PS I remains at the interface, and that the loss of PS I, due to dissolving into the subphase, is not significant [2].

In situ absorption spectra for PS I monolayers on different concentrations of NaCl subphase surface was also investigated (Figures not shown). The results indicate that no obvious band can be recorded for PS I monolayer on the surface of NaCl subphase with its concentration above 1 mol/L, which may be attributed to that the protein, PS I, becomes unstable on the surfaces containing high concentrations of salt.

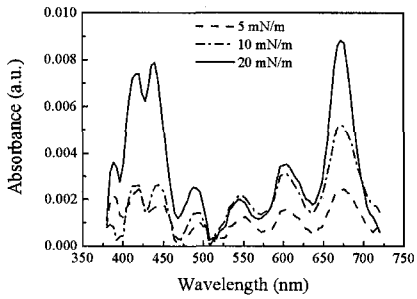


Figure 2. ***In situ*** **absorption spectra of PS I on the 0.1 mM PBV subphase surface.**

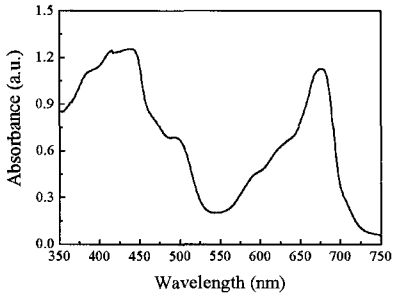


Figure 3. PS I absorption spectrum in solution.

Langmuir-Blodgett films of PS1

The monolayers of PS I were difficult to be transferred onto hydrophilic substrate surface, also difficult to be transferred by using a vertical dipping method. Successful deposition can be performed by a horizontal lifting method on hydrophobic substrate surfaces. Figure 4 shows the absorption spectra of PS I LB films transferred from the 0.5 M NaCl, 0.02 M $CaCl_2$, 0.1 mg/mL PDDA and 0.1 mM PBV subphase surfaces. All curves are composed of two main absorption bands at about 420~440 and 675 nm, corresponding to the chlorophyll a in the PS I. The spectra do not show significant difference from that in buffer solution, which may suggest that the PS I is not denatured after the monolayer transfer [2]. A comparison with the absorption in PS I solution indicates that the shoulder band at about 416 nm is slightly weakened in the LB films, but no obvious change is observed for the absorption band at 440 nm. On the other hand, the absorption band at 676 nm red shifts 2~3 nm for the LB films deposited from NaCl and $CaCl_2$ subphase surfaces, while blue shift 2~3 nm for the LB films from polymer subphase surfaces. These may be ascribed to that the adsorbed cationic ions or polymers slightly affect the aggregation of PS I in the LB films.

The complex PS I multilayers can be obtained by the horizontal lifting method with well washing the substrate after each transfer. Figure 4d shows absorption spectra of five layers of PS I/PBV multilayer. Absorptions at~440 nm and~674 nm increase with the increase of layer numbers. Moreover, this multilayer exhibits quite strong stability in buffer solution. As shown in Figure 5, over 80 % PS I still remains in the multilayer after the PS I LB film coated substrate was immersed in the buffer for 1 h.

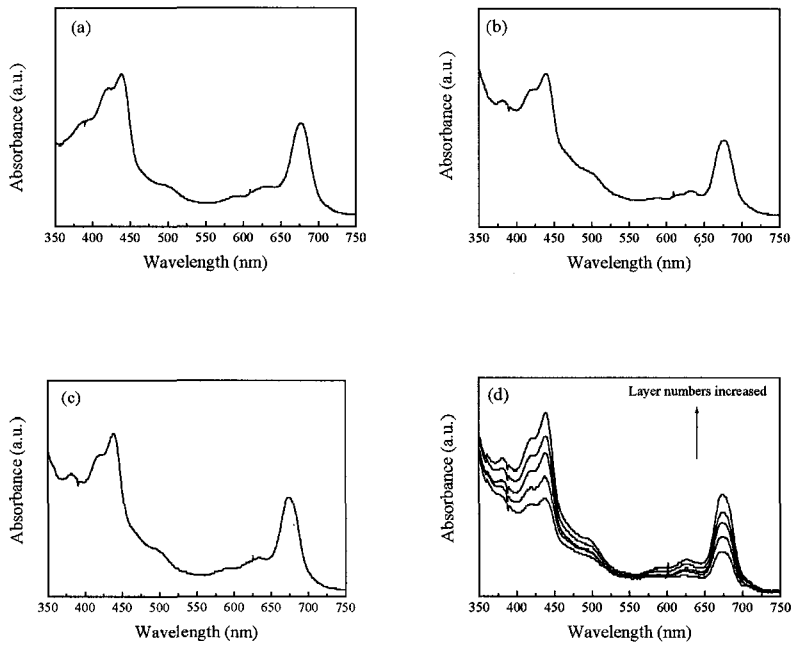


Figure 4. Absorption spectra of PS I LB films from (a) 0.5 M NaCl, (b) 0.02 M $CaCl_2$, (c) 0.1 mg/mL PDDA, and (d) 0.1 mM PBV subphase surfaces.

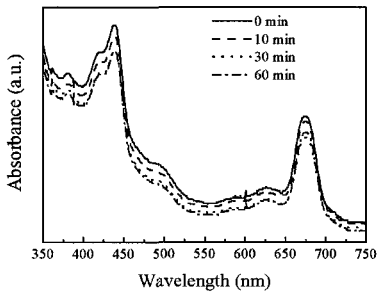


Figure 5. Absorption spectra of PS I/PBV LB film after immersed in MES buffer.

QCM response of the PS I LB monolayer

At 9 MHz oscillation frequency, the measured frequency change (Δf) was about 244, 170, 125 and 166 Hz after the transfer of PS I LB monolayer from the 0.5 M NaCl, 0.02 M $CaCl_2$, 0.1 mg/mL PDDA and 0.1 mM PBV subphase surfaces, respectively. Based on these ΔF values, we can get a mass change (Δm) of about 260, 181, 133 and 178 ng/cm^2, respectively, according to the equation

$$\Delta F = -2F_0^2\Delta m/(A\cdot\rho_q^{1/2}\cdot\mu_q^{1/2}) \qquad (1)$$

where F_0 is the fundamental resonant frequency of 9 Hz, Δm (g) is the mass change, A is the electrode area (0.196 cm^2), ρ_q is the density of the quartz (2.65 g/cm^3), and μ_q is the shear module (2.95×10^{11} dyne/cm^2) [13]. These mass changes are quite larger than those for monolayers of organic amphiphiles with one or two long alkyl chains [14, 15], suggesting that the large PS I unit is transferred onto the gold surface.

Cyclic voltammogram of PS I-PBV complex film

Since viologen derivatives can act as electron carrier for PS I, a study of a complex film of PS I with viologens is interesting in the development of biomolecular device [6]. Figure 6 shows a CV curve of PS I/PBV complex LB film at a scan rate of 100 mV/s in 10 mM KCl electrolyte solution. Two reversible redox waves were recorded with reduction potentials at about -650 and -1050 mV vs Ag/AgCl, which correspond to the monoelectronic process of $PBV^{2m+} \leftrightarrow PBV^{m+\cdot}$ and $PBV^{m+\cdot} \leftrightarrow PBV^0$, respectively [16]. These results indicate that the PBV molecules are adsorbed by the PS I monolayer. Compared to previous data for small viologen amphiphile LB films [16], the reduction potentials of PS I/PBV LB film shift negatively and oxidation potentials shift positively. This shift can be ascribed to the fact that the large protein unit becomes an electron barrier for viologen electrochemical reaction.

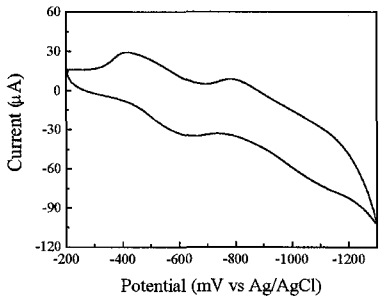


Figure 6. Cyclic voltammograms of PS I/PBV LB film modified electrode.

CONCLUSIONS

Cationic ions and polyelectrolytes can stabilize the formation of the PS I monolayers at the air-water interface. These complex monolayers can be transferred onto the hydrophobic substrate surfaces by horizontal lifting method. The PS I/polyelectrolyte complex film may be used for the development of a biosystem for the studies on photoinduced electron transfer and for hydrogen evolution.

ACKNOWLEDGEMENT

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MODULAR DEVICE FOR HYDROGEN PRODUCTION : OPTIMIZATION OF (INDIVIDUAL) COMPONENTS

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ABSTRACT

In order to create a semiartificial device for hydrogen production, several components have to be optimized. Here we report about the construction of His-tagged PS1 and PS2 which should speed up and facilitate isolation of these reaction centers and also enable an oriented immobilization on the electrode surfaces of the device. Properties of isolated His-tagged PS1 and PS2 are compared with their WT counterparts in detail in order to find out whether there is an influence of the His-tag on purity, activity and heterogeneity.

KEYWORDS:

Hydrogen production, water-splitting photosynthesis, semiarteficial system, His-tagged membrane proteins, photosystem 1, photosystem 2

INTRODUCTION

Long-term aim of our project is the construction of a biomolecular device for hydrogen production in combination with light-driven water-splitting as it occurs in the natural process of photosynthesis in plants. Such a semiartificial device should combine the best suited components found in various native systems which – up to now – cannot be found in an individual native system due to incompatibilities and/or different origin (pro- and eukaryotic, meso- and thermophilic, aerobic – anaerobic environment). Advantage of such a system is

a) it is modular, i.e. all components can be easily exchanged
b) the separate development and optimization of all components is possible
c) the regeneration of the key components in native systems is possible
d) the highest possible stability of all components can be realized

These most-suited components should be arranged on the surface of electrode materials to finally achieve light-driven hydrogen production. To avoid the recombination of oxygen with hydrogen and at the same time the (ir-)reversible inhibition of the hydrogenase by oxygen, we choose two separate reaction chambers which are connected by a salt bridge and a

conducting wire. While the left chamber in this model-set-up (Fig. 1) contains oxygen due to the process of water-splitting in light, performed by photosystem 2 (PS2), the right chamber is kept under anaerobic conditions to guarantee optimal performance of the oxygen-sensitive hydrogenase. A separate long-term branch of this project should contribute to improve the oxygen-tolerance of hydrogenases by using directed and random mutagenesis approaches.

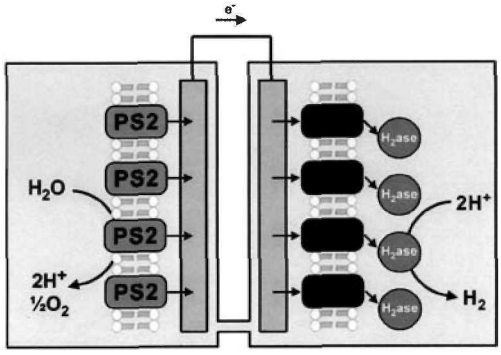

Figure 1. Model of the hydrogen device with the left chamber operating under aerobic conditions (due to water-splitting of PS2) and the right chamber under anaerobic.

MATERIALS AND METHODS

Construction of His-tagged PS1-subunit psaF and PS2-subunit psbB

Cloning of constructs was performed according to standard protocols [Sambrook et al. 1989]. The mutations were created in the plasmid pBluescript II KS (+) by oligonucleotide-directed mutagenesis.

The mutagenic primer, 5'CGCGGATCCCATCATCATCACCATCATCACCATCATCACG CTAGCGATGTGGCAGGACTCGTCCCTTGCAAAGAT-3', was used to fuse10 histidyl-codons to the start of the *psa*F-gene. The resulting plasmid pHispsaF contained the gene sequence, the His-tag and an additional upstream-region in which a chloramphenicol resistance was introduced for selection.

A similar strategy was used to create plasmid pCP47. The insertion of a 10-His-tag was realized by cloning the tag at the end of the *psb*B gene via the restriction-sites *Bam*HI and *Eco*RV (see Fig. 2). In case of plasmid pCP47His, the selection-marker Cm^R was located downstream of *psb*B.

Transformation of Thermosynechococcus elongatus BP-1

Transformation of plasmids pHispsaF and pCP47 into *Thermosynechococcus elongatus* (*T. elongatus*) cells was carried out by electroporation.

Selection of mutants was done by increasing the chloramphenicol concentration in liquid BG-11. Control and mutant strains were grown at a light intensity of 30 µmol photons·m^{-2}·s^{-1} at 45°C with air/CO_2 bubbling.

Isolation of PS2 core complexes and activity measurements

Unmodified PS2 core complexes highly active in oxygen evolution were isolated by a two-step HPLC procedure according to [Kuhl et at. 2000], with some minor modifications as specified in [Nowaczyk et al., in this book]. For the preparation of His-tagged PS2, thylakoid membranes were solubilized in 20 mM MES pH 6.5 / 10 mM $CaCl_2$ / 10 mM $MgCl_2$ / 200 mM $(NH_4)_2SO_4$ / 1.2% ß-DM / 0.5% Na-cholate at a *Chl* concentration of 1 mg/ml. After centrifugation at 45.000 × *g* for 90 min at 4°C, the supernatant was loaded onto a chelating sepharose fast flow column (Pharmacia) which had been equilibrated with 50 mM MES pH 6.5 / 10 mM $CaCl_2$ / 10 mM $MgCl_2$ / 300 mM NaCl / 10% glycerol / 0.02% ß-DM / 1 mM imidazole (equilibration buffer). The column was then flushed with 4 volumes of equilibration buffer at a flow rate of 2 $ml \cdot min^{-1}$. PS2 core complexes were eluted with a linear gradient from 1 mM to 200 mM imidazole and dialysed over night against 20 mM MES pH 6.5 / 10 mM $MgCl_2$ / 10 mM $CaCl_2$ / 0.5 M mannitol / 0.03% ß-DM. To separate PS2 monomers and dimers the resulting protein solution was loaded on a second column (IEC) as in [Kuhl et at. 2000]

PS2 activity measurements were done with an oxygen sensor consisting of a polymer optical fibre (POF) coated with an oxygen-sensitive foil (Presens, Regensburg, Germany) as specified in [Nowaczyk et al., in this book].

Isolation of PS1 core complexes and activity measurements

Preparation and isolation of His-tagged PS1 by a chelating sepharose fast flow column (Pharmacia) were done as indicated above (see previous subsection). In order to separate highly enriched His-tagged PS1 trimers, the protein solution was mixed 1:1 with ammonium sulfate high salt buffer (20mM HEPES pH 7.5 / 10 mM $MgCl_2$ / 10 mM $CaCl_2$ / 3 M $(NH_4)_2SO_4$) and loaded on a second column (hydrophobic interaction, HIC) which had been equilibrated with a high salt buffer (20 mM HEPES pH 7.5 / 10 mM $MgCl_2$ / 10 mM $CaCl_2$ / 1.5 M $(NH_4)_2SO_4$ / 0.03% ß-DM). The column was flushed with 4 volumes buffer at a flow rate of 3 ml/min. The PS1 core complex was eluted with a linear gradient from 1.5 M to 0 M ammonium sulfate (in total 4 column volumes).

PS1 activity measurements were done by an oxygen sensor at 30°C (see previous subsection). 5 µg sample was mixed with HEPES buffer (0.03 M HEPES pH 7.0 / 0.003 M $MgCl_2$ / 0.05 M KCl / 0.33 M Mannitol), 0.2% ß-DM / 0.08 mM DCPIP / 0.5 mM Na-ascorbat / 0.5 mM methylviologen / 0.01 mM DCMU / 2 µM Na-azide).

SDS-PAGE analysis

PS1 and PS2 core complexes were solubilized by 1% SDS and separated by SDS-PAGE using a 12 % gel containing 6 M urea according to[Schägger and von Jagow 1987].

Analytical size exclusion chromatography

Analytical size exclusion chromatography was performed on a Waters HPLC system using a Biosil SEC-400 column (Biorad) acc. to [Kuhl et at. 2000] in order to examine the oligomerization status of PS1 and PS2.

RESULTS

Construction of His-tagged PS1 and PS2 from Thermosynechococcus elongatus

In order to be able to isolate both PS1 and PS2 from the thermophilic cyanobacterium *Thermosynechococcus elongatus* by a one-step procedure and in order to immobilize both photosystems on the surface of the support materials (i.e. the electrodes) in an oriented

manner, *His-tags* have been fused to the genes of appropriate subunits of these complexes. Care was taken to select sites which guarantee that – according to the model shown in Figure 1 – the acceptor side of PS2 and the donor side of PS1 are equipped with the *His-tag* in order that this site is oriented towards the electrode surface upon binding. In order to improve the probability for binding of the *His-tag* to the column matrix – despite various loops of these membrane complexes exposed to their hydrophilic surfaces –a 10-*His-tag* instead of a 6-*His-tag* has been chosen. Due to the structural information available for both photosystems [Schubert et al. 1997; Zouni et al. 2001] the genes encoding the following subunits have been selected for His-tag attachment:

A. The C-terminus of PsbB (CP47) was chosen according to[Bricker et at.1998]; this report showed that the isolation of a highly active PS2 from *Synechocystis* sp. PCC 6803 using a *His-tagged* mutant of CP47 was possible.
B. Structural data of PsaF led to the conclusion that the N-terminus of the protein is located on the donor side of PS1. Another reason for choosing the N-terminus of PsaF is the fact that *psa*F and *psa*J gene are organized in one operon and therefore insertions between the two genes might lead to a functional disorder [Xu et at.2001].

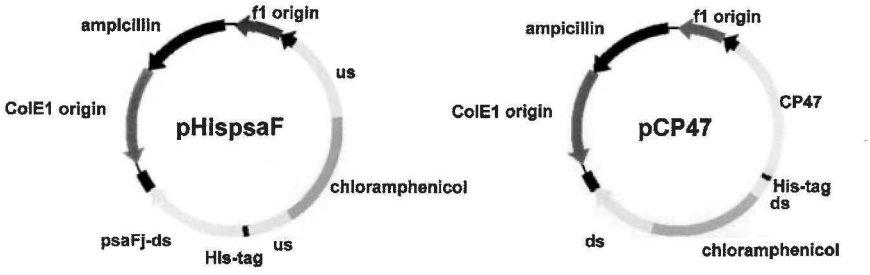

Figure 2. Constructs of His-tagged PS1 + PS2.
a) Vector map of pHispsaF. Vector elements: *psa*FJ-DS: *psa*FJ-operon + downstream region (DS); His-tag: 10-Histidine-tag; US: upstream-region
b) Vector map of pCP47. Vector elements: *psb*B; His-tag: 10-Histidine-tag; downstream region (DS)

Purification of the His-tagged Photosystems

His-tagged PS1 and PS2 were purified in a first step on an IMAC-column (chelating sepharose fast flow column, Pharmacia, see M&M) as shown for PS2 in Figure 3; especially free pigments and unwanted proteins were separated by this step. Purification of PS1 was done accordingly and resulted in a similar elution profile (data not shown). Routinely, after this column PS2 was further purified on an anion exchange column, while PS1 was further purified by a hydrophobic interaction chromatography step acc. to [Wenk and Kruip 2000] (data not shown). Both procedures resulted in preparations of increased purity and homogeneity as shown by HPLC size exclusion chromatography and SDS-PAGE analysis: Figure 4 (upper part) shows the elution profile of PS1 from a size exclusion column; comparison with marker proteins indicates that PS1 is nearly exclusively isolated as stable

trimeric complex. SEC also shows (Fig. 4, lower part) that PS2 is mainly isolated as a dimer, with a smaller peak of PS2-aggregates and a shoulder indicating some PS2 monomers. The purity of both complexes was analysed by SDS-PAGE (Fig. 5). The subunit pattern of PS1 indicates the presence of many impurities after the IMAC column (track 1), which are removed by the second column step. i.e. the HIC-HPLC (track 2).

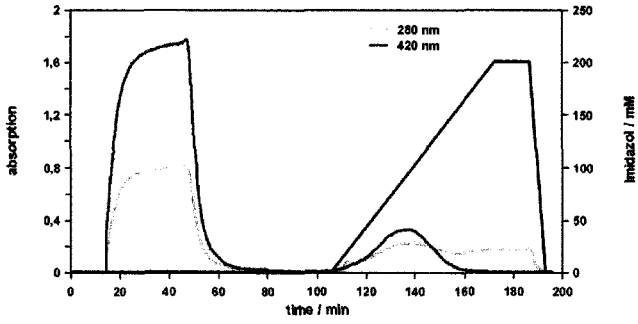

Figure 3. Purification of PS2-His-tagged on a chelating.

In case of PS2, the purification by the second column step, i.e. the IEC, is not so obvious in comparison to the second column step of PS1 (compare track 1 and 2 of PS2). However, for PS2 this step is necessary to separate monomeric from dimeric PS2 with the latter being prefered for our hydrogen device due to higher activity and longer stability [see also Kuhl et al. 2000]. For both PS1 and PS2, the known subunits of each complex (as indicated on the right side of the gel) have been identified by homology with bands from WT preparations, by specific antibodies or by mass spectroscopy (data not shown). In general, all major bands of both complexes and – as far as identified – all small subunits could be preserved in our *His-tagged* preparations in comparison with gels of WT-PS1 [Wenk and Kruip 2000] and WT-PS2[Kuhl et al. 2000]. In conclusion, SDS-gels after the second column do not display major unknown proteins nor the loss of major subunits of the PS1- and PS2-complex, respectively.

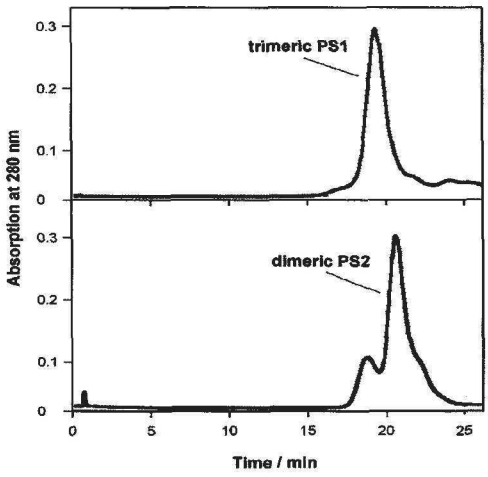

Figure 4. Size-exclusion chromatography of purified PS1 and PS2.

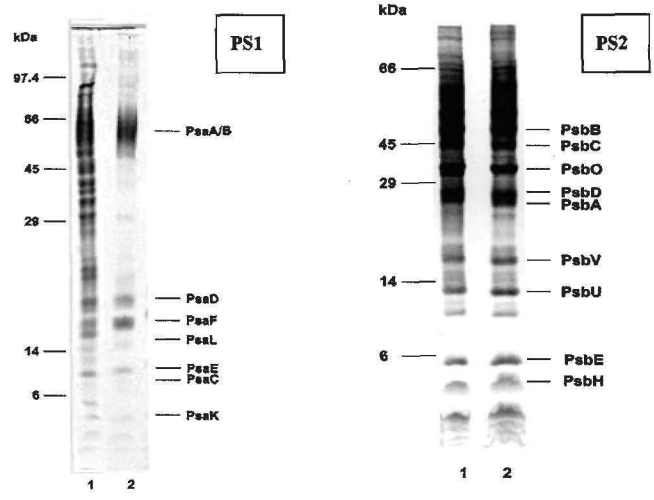

Figure 5. SDS-gel of purified PS1-His-tag (left) and PS2-His-tag (right) after IMAC-column (1) and after HIC-column (PS1) or IEC-.

Table 1 compares the specific activities of PS1 and PS2, respectively, in comparison with PS1- and PS2 complexes isolated from WT. These data show, that the activities of *His-tagged* complexes are only slightly lower than the respective WT complexes, i.e. the *His-tag* in these complexes apparently does not interfer with the activity under these conditions.

Table 1. Activity of PS1 and PS2-His-tag in comparison with untagged PS1 and PS2, respectively

	WT-PS2	His-tagged PS2		WT PS1	His-tagged PS1
Activity / $\mu mol\ O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$	≤ 2,300	≤ 2,200	Activity / $\mu mol\ O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$	≤ 1,000	≤ 820

DISCUSSION AND OUTLOOK

His-tagged photosystems

PS2: Our PsbB-His-tagged PS2 construct is similar to the one constructed before for the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 Bricker et al.[Bricker et al. 1998] with the only difference that we used 10 instead of 6 histidine residues in order to achieve a more efficient purification of the tagged photosystem. The only report about a thermophilic PS2 attached a 6-His-tag on the CP43 subunitSugiura AND Inoue [Sugiura and Inoue 1999] In contrast to this report, our PS2 eluted at a considerably lower Imidazole concentration from the IMAC-column, i.e. about 40 mM instead of 200 mM. This may indicate that – in spite of the larger number of histidine residues on the histidine-tag – our PS2 is less tightly bound to the column matrix, which in turn maybe due to a decreased accessibility of the His-tag on the stroma-side of the CP47 subunit in comparison to the CP43 subunit.

Due to continuous improvement of the isolation conditions, our preparation now shows similar activity rates for light-induced oxygen formation as the dimeric PS2 preparation from WT. The high activity of our preparation is also reflected by the high amount of dimeric PS2 as shown by size exclusion chromatography and by the presence of the PsbO-subunit which is supposed to shield the Mn-complex. Major difference in comparison with WT-PS2 is a higher sensitivity towards basic pH (above pH 7.5, unpublished observation); this, however, is no drawback for our purpose as the PS2-compartment of the hydrogen device operates at lower pH under all conditions. On the other hand, major advantage of this *His-tagged* PS2-preparation is the considerably shorter preparation time: Even if a second purification step is included (i.e. the IEC-column), the total time for isolation and purification is less than 50% in comparison to the WT PS2-preparation.

PS1: The PS1-prep. introduced in this communication is the first reported with a *polyhistidine tag* fused to the N-terminus of the PsaF subunit. This construct was possible due to the fact that cyanobacterial PsaF-deletion mutants show no impact on photoautotrophic growth – in contrast to *Chlamydomonas reinhardtii*, where inactivation of PsaF results in a severe reduction of electron transfer from plastocyanin to PS1[Hippler et al. 1997]. Also, the N-terminus of the F-subunit which was decorated by the *tag* is located towards the lumen side which enables an attachment of the isolated PS1 with the lumen-exposed /donor-side to the electrode surface in our hydrogen-producing device.

Due to problems concerning the isolation of single *Thermosynechococcus*-colonies the segregation process of both described *His-tag* mutants was not complete. These problems have been solved now and it is likely that a complete segregation of both mutants will even more increase the amount of isolated PS1 and PS2 complex.

In summary, the experiments reported in this contribution now allow the following set-up of the device:

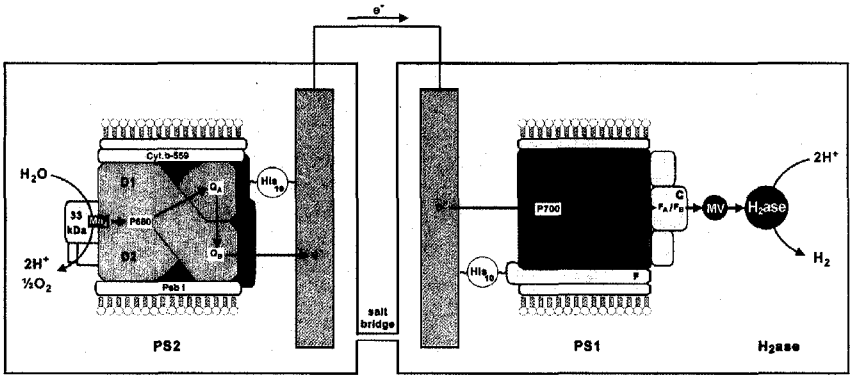

Figure 6. Model of the hydrogen producing device, illustrating location and orientation of *His-tagged* PS1 and PS2 as engineered in this report as part of the semiartificial electron transport chain for the production of hydrogen from water.

Our experiments are now focused at optimizing conditions to immobilize as much photosystems as possible on appropriate electrode surfaces. Also, mobile or immobilized electron donors and acceptors have to be evaluated for an optimal electron flow to and from the electrodes. Having achieved already an efficient immobilization of hydrogenase on electrode surfaces which resulted in hydrogen production [Qian et al. 2002; Wenk et al. 2002], the combination of all these approaches should finally lead to the construction of a hydrogen producing device as outlined in the introduction section and extend our knowledge on light-triggered hydrogen production from water as a future energy source.

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V. Appendices

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