II.D.3 Algal H_2 Production Systems: Creation of Designer Alga for Efficient and Robust Production of H_2

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Objectives

Develop advanced renewable photobiological hydrogen production technologies through creation of a designer alga by genetic insertion of a proton channel into algal thylakoid membrane. By 2015, demonstrate an engineering-scale biological system that produces hydrogen at a plant-gate cost of $10/kg.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

- J. Rate of Hydrogen Production

Approach

Overcome the technical barrier by creating a designer alga through genetic insertion of a proton channel into algal thylakoid membrane for significant (10-fold) improvement in photobiological hydrogen production from water.

Accomplishments

- Performed bioinformatics analyses on natural ionophores: the brown adipose tissue uncoupling proteins (UCP-1 and UCP-2) and melittin for design of the envisioned proton channel to enhance algal hydrogen (H_2) production.
- Maintained valuable Oak Ridge National Laboratory (ORNL) algal culture stocks, instruments, and R&D capabilities.

Future Directions

- Design and construction of DNA sequence coding for polypeptide proton channel.
- Genetic transfer of hydrogenase promoter-linked polypeptide proton-channel DNA into algal strain DS521.
- Characterization and optimization of the polypeptide proton-channel gene expression.
- Demonstration of efficient and robust production of H_2 in designer alga (ready for next phase: scale-up and commercialization).

Introduction

Algal (such as *Chlamydomonas reinhardtii*) photosynthetic H_2 production from water has tremendous potential to be a clean and renewable energy resource. However, there are a number of technical issues that must be addressed before algal H_2 production can become practical. The goal of this project is to overcome a major technical barrier: rate of hydrogen production. This technical barrier currently consists of four physiological obstacles that seriously limit the absorbed light energy to hydrogen efficiency (E1*E2). These four physiological obstacles that currently challenge researchers and investors in the field of photosynthetic H_2 production are (1) restriction of photosynthetic H_2 production by accumulation of a proton gradient, (2) competitive inhibition of photosynthetic H_2 production by CO_2, (3) requirement of bicarbonate binding at photosystem II (PSII) for efficient photosynthetic activity, and (4) competitive drainage of electrons by O_2 in algal H_2 production. In order for the photobiological H_2 production system to work, all of these four problems must be solved. Therefore, in this project, we will overcome these four technical problems by creating an efficient and robust algal H_2 production system through a new and novel approach that has recently been developed at ORNL. In this approach, a "designer alga" will be created by genetic insertion of hydrogenase promoter-programmed polypeptide proton channels into algal thylakoid membranes. This approach will allow us to simultaneously solve the four physiological problems because all four problems are proton gradient-related. Success of this work will have a significant impact (a 10-fold improvement) on technology development in the field of renewable hydrogen research.

Approach

We have recently developed a systematic approach to create a "super" photosynthetic organism – a "designer alga" that is specifically designed for production of molecular hydrogen through photosynthetic water splitting.[1] This designer alga will be able to overcome the four proton gradient-related physiological problems (listed above) that currently challenge researchers and investors in the field of photosynthetic H_2 production. The key element of our proposed approach is creation of a designer alga for efficient and robust production of H_2 through genetic insertion of a programmable polypeptide proton channel into the thylakoid membrane. The genetic insertion of programmable proton channels into a thylakoid-membrane is proposed to be achieved by transformation of a host alga with a genetic vector that contains a polypeptide proton-channel gene linked with a hydrogenase promoter. The envisioned super alga that can be created by the proposed work should be able to perform autotrophic photosynthesis using ambient-air CO_2 as the carbon source and grow normally under aerobic conditions such as in an open pond. When the algal culture is grown and ready for H_2 production, the proton-channel gene will then be expressed simultaneously with the induction of the hydrogenase enzyme under anaerobic conditions because of the use of the hydrogenase promoter. The expression of the proton-channel gene should produce polypeptide proton channels in the thylakoid membrane, thus dissipating the proton gradient across the thylakoid membrane without adenosine triphosphate (ATP) formation to enhance H_2 production.

As illustrated in Figure 1, our recent experimental studies with the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (FCCP) have already demonstrated that insertion of a proton-conductive channel in the thylakoid membrane could significantly enhance H_2 production by eliminating the problems of both the proton gradient accumulation and the newly discovered alternative O_2 sensitivity that is dependent on the proton gradient.[2] Furthermore, the cessation of photophosphorylation (ATP formation) caused by action of the proton channels can, in turn, switch off the Calvin cycle activity (CO_2 fixation), which requires ATP and competes with the ferredoxin/hydrogenase H_2 production pathway for the photosynthetically generated electrons. As a result, the competitive inhibition of H_2 production by CO_2 will now be eliminated, and photosynthetic H_2 production in the designer alga will be able to occur in the presence of CO_2. Since photosynthetic H_2 production in a successful designer alga no longer requires a CO_2 (HCO_3-)-free environment, the requirement for HCO_3- binding at photosystem II (PSII) for efficient photosynthetic activity will also

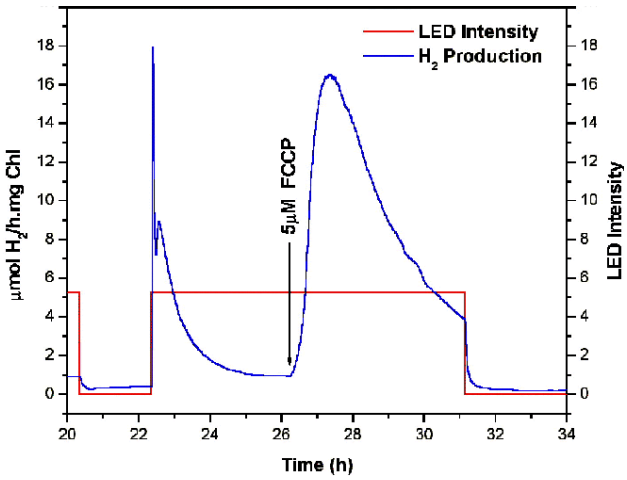

Figure 1. Stimulation of photosynthetic H_2 production in *C. reinhardtii* 137c following addition of the proton uncoupler FCCP in a background atmosphere of 1000 ppm O_2. Addition of 5 μM FCCP produced a dramatic increase in H_2 production, followed by a slow decay. The slow decay is due to a side-effect of FCCP, known as ADRY,[3] in which FCCP gradually inhibits PSII activity. This ORNL experimental result indicates that use of a polypeptide proton channel that does not have the ADRY effect could enhance H_2 production by eliminating the problems of both the proton-gradient accumulation and the newly discovered alternative O_2 sensitivity.

no longer be a problem. The requirement can now be satisfied by leaving some CO_2 in the medium.

Results

In addition to the effort of maintaining the ORNL algal H_2 R&D capabilities and facilities, including its valuable algal culture stocks and the dual-reactor-flow H_2 detection system, we have now performed some bioinformatics analysis to design DNA sequences for the envisioned proton channel. In our computational analysis for the design of the proton channel, we also included the structures of the "brown adipose tissue peptide (uncoupling protein)" and "melittin" that the reviewers at the FY 2003 DOE Hydrogen Program Merit Review recommended for inclusion in our research. According to the merit review that we received from the DOE Office of Energy Efficiency and Renewable Energy Hydrogen Program in October 2003, the

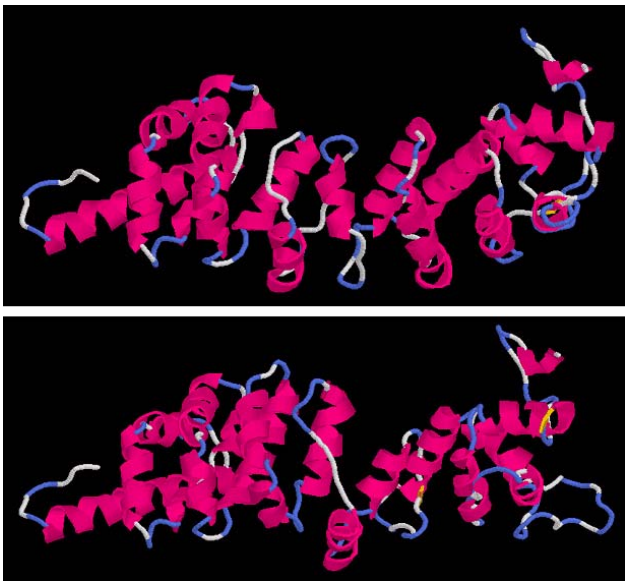

Figure 2. The Structures of Brown-Adipose-Tissue Uncoupling Proteins UCP-1 (top) and UCP-2 (bottom) that We Have Analyzed with ORNL's PROSPECT Computer Software Tools

reviewers clearly understood and support our proposed designer alga H_2 production technology concept and commented that our "approach is novel, sound and even exciting." They also commented that we should also work on natural ionophores, such as the "brown adipose tissue peptide" and perhaps "melittin" in addition to our "synthesis" approach. Therefore, using ORNL's unique computer software (PROSPECT), we have now analyzed the DNA sequences and the structures of both "brown adipose tissue peptide" (Figure 2) and "melittin" (Figure 3). Based on our analysis and the available literature, the "brown adipose tissue peptide" that our reviewers referred to is the uncoupling protein (UCP) in mammalian brown adipose tissue (BAT) mitochondria. Three isoforms of UCP have now been identified in the mammalian system: UCP-1, UCP-2 and UCP-3. UCP-1, which is the best known of the three, consists of 306 amino acids with a calculated molecular mass of 33.2 kDa. UCP-1 is located in the BAT inner mitochondrial membrane. It can catalyze proton re-entry into the matrix and thus dissipate the proton electrochemical potential gradient as heat. Consequently, UCP-1 plays a significant role in thermogenesis, and its activity is physiologically regulated (e.g., purine nucleotides inhibit while fatty acids activate its activity for

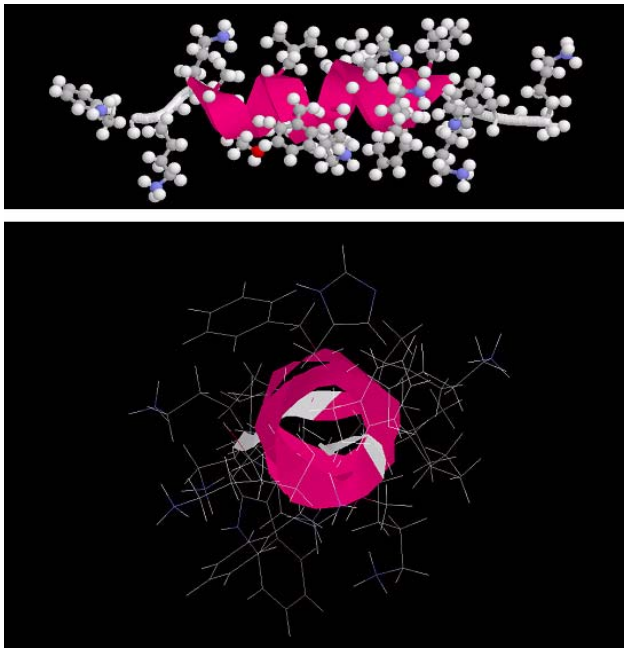

Figure 3. The side view (top) and perpendicular view (bottom) of melittin structure that we have analyzed with ORNL's PROSPECT computer software tools. Melittin can conduct protons without the complicated regulation mechanisms that UCPs require. The structure of melittin is quite similar to our preliminary design of the proton channel (Figure 4) that was based on an analogue of gramicidin *A*.

proton transport) in the tissue. Recently, a number of UCP homologues have been discovered not only in mammals (e.g., the three isoforms of UCP: UCP-1, UCP-2 and UCP-3), but also in certain green plants, such as the UCP homologue recently identified in *Arabidopsis thaliana* (AtUCP).[4] The UCPs are evolutionarily related and possibly derived from a proton/anion transporter ancestor. Their DNA sequences are also known. Therefore, we can and will use them as a reference in our design for the envisioned proton-channel gene to enhance algal photobiological H_2 production.

Melittin is an amphipathic peptide of 26 amino acids with primary sequence GIGAVLKVLTTGLPALISWIKRKRQQCONH$_2$. The peptide is the major toxin in honeybee (*Apismellifera*) venom. As shown in Figure 3, we have now also analyzed its known structure by computer. According to our analysis, melittin is probably better than UCPs for our application

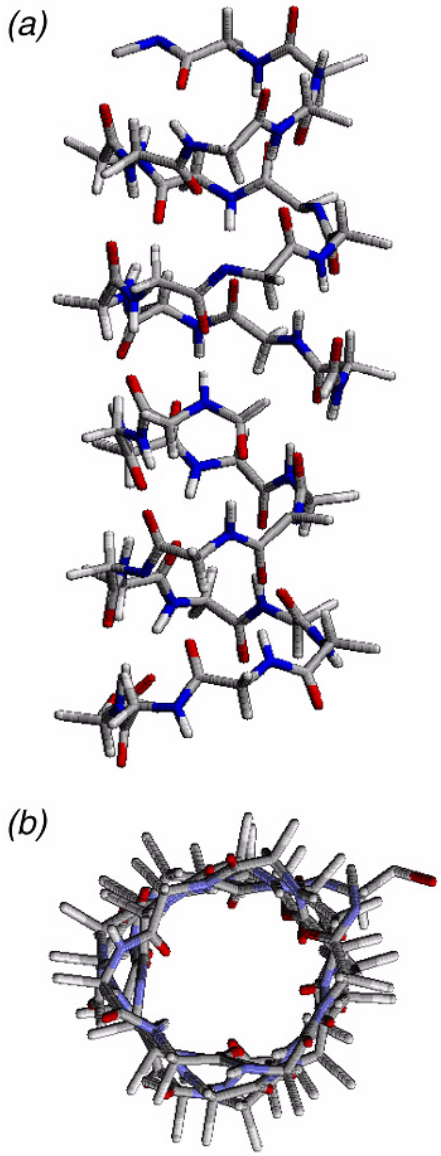

Figure 4. A preliminary design of a polypeptide proton channel achieved by computer simulations using the structural information of gramicidin A at ORNL. The side view **(a)** and top view **(b)** of the preliminary proton-channel design are presented here. Future work is needed to optimize and complete the design to achieve the envisioned genetic insertion of polypeptide proton channels into the algal thylakoid membranes for enhanced photobiological H_2 production.

because it is much simpler than the UCPs. Melittin can conduct protons without the complicated regulation mechanisms that UCPs require. The structure of melittin is also quite similar to our

preliminary design of the proton channel (Figure 4) that was based on an analogue of gramicidin *A*. Therefore, our further work will focus on application of melittin-like structure to achieve our envisioned genetic insertion of polypeptide proton channels into the algal thylakoid membranes to significantly enhance the rate of electron transport for photobiological H_2 production.

Conclusions

Creation of designer algae by genetic insertion of proton channels into thylakoid membranes is one of the key R&D tasks that are required for the photobiological H_2 production system to work. We have already developed a systematic approach to achieve the proposed work. The proof of principle for this designer alga H_2 production R&D project has been demonstrated through the FCCP experiments. A preliminary design of a polypeptide proton channel has been achieved through bioinformatics computer simulations. Future work is needed to finalize the proton-channel design and construct the needed synthetic gene to achieve the envisioned genetic insertion of polypeptide proton channels into the algal thylakoid membranes for enhanced photobiological H_2 production.

References

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3. Samuilov, V. D., E. L. Barsky, and A. V. Kitashov (1995) "ADRY agent-induced cyclic and non-cyclic electron transfer around photosystem II," *Photosynthesis: from Light to Biosphere*, P. Mathis (ed.), Vol. II, 267–270. The Netherlands, Kluwer Academic Publishers.

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2. Lee, James W., Laurie Mets, Dong Xu, Barbra Evans, and Jizhong Zhou. "Development of efficient ad robust algal hydrogen production systems," presented at the DOE Hydrogen, Fuel Cells & Infrastructure Technologies Program Merit Review meeting, May 19-22, 2003, Berkeley, California.

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