Algal Systems for Hydrogen Photoproduction

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Objectives

- Generate algal hydrogenase mutants with higher O_2 tolerance to function with aerobic H_2-production systems being developed in collaboration with Oak Ridge National Laboratory (ORNL) and University of California (UC) Berkeley.
- Optimize the rates of H_2 production using an anaerobic production system discovered by UC Berkeley and NREL.

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 R,D&D Plan:

- K. Continuity of Photoproduction

Approach

Subtask 1 (Hydrogenase Function and Molecular Engineering):

- Apply different mutagenesis techniques to generate O_2-tolerant algal hydrogenases.
- Select transformants that express recombinant hydrogenases and study their H_2-production capability in the presence of varying amounts of added O_2.
- Genetically combine the O_2-tolerance phenotype with truncated antenna (UC Berkeley) and proton channel (ORNL) mutants.

Subtask 2 (Biochemical and Process Characterization):

- Develop a sulfur-deprived system for continuous H_2 photoproduction.
- Optimize engineering and biochemical parameters.
- Demonstrate continuous H_2 production for a total of 800 hours by 2008.

Accomplishments

- By homology modeling, identified residues in algal hydrogenase HydA1 that form the 5.5-7.0 Å diameter gas channel and mapped them to three regions: close to the catalytic site, mid-way to the surface, and close to the surface (subtask 1).
- Mutagenized one of the residues located next to the catalytic site (valine 240) and demonstrated H_2 production with increased tolerance to O_2 (subtask 1).
- Developed a system for continuous H_2 production based on a two-reactor, sulfate-chemostat design (subtask 2).

- Demonstrated continuous H_2 production for a total of 480 hours using the new photobioreactor system (subtask 2).

Future Directions

- Perform molecular dynamics simulation to validate our results with the O_2-tolerant mutant and guide further site-directed mutagenesis work.
- Generate and test multiple algal hydrogenase mutants by a variety of mutagenesis techniques (e.g., site-directed, random *in vitro*, and gene shuffling).
- Accelerate the identification of O_2-tolerant clones by developing new high-throughput assays.
- Use spectroscopic and biochemical techniques to investigate and correct or circumvent the rate limitations inherent to the sulfur-deprived system.
- Examine alternative photobioreactor designs.
- Increase the duration of H_2 production (1,500 h at >2 ml/h in 2010) using a mutant that incorporates the best properties of the NREL, UC Berkeley and ORNL individual mutants.

Introduction

The green alga, *Chlamydomonas reinhardtii*, has the biochemical machinery necessary to efficiently photoproduce H_2 from water. However, under normal conditions where O_2 is a by-product of photosynthesis, sustained algal H_2 photoproduction cannot be maintained for more than a few minutes (Ghirardi et al., 1997). Oxygen is a powerful inhibitor of algal [Fe]-hydrogenase (the catalyst that releases H_2 gas) function, and H_2 photoproduction stops abruptly if O_2 is present. Oxygen inactivation is thought to occur by the direct binding of O_2 to one of the iron species (with an unoccupied coordination site) located at the catalytic center (Adams, 1990). Our current project addresses two different strategies for surmounting the O_2-sensitivity problem. These include (a) molecular engineering of the hydrogenase to remove the O_2 sensitivity (subtask 1) and (b) development of physiological means to separate O_2 and H_2 production (subtask 2).

Our early work for the DOE Hydrogen Program involved the cloning and sequencing of two algal hydrogenase genes (Forestier et al., 2001, 2003), and this provided us with the tools and background knowledge to make rapid progress in the molecular engineering area. The work on subtask 1 has focused on a knowledge-based approach and is addressing regions in one of the algal hydrogenases (HydA1) that could be involved in access of O_2 to the catalytic site. This annual report describes the method that we used to (a) identify this region and (b) design, generate and test the first site-directed mutant targeted to improved O_2 tolerance.

In the absence of an O_2-tolerant hydrogenase, we demonstrated (with UC Berkeley) that it is possible to physiologically manipulate *C. reinhardtii* to generate anaerobic conditions, induce the hydrogenase gene and photoproduce H_2 sustainably for about 3-4 days (Melis et al., 2000; Ghirardi et al., 2000). This method was based on the partial inactivation of photosynthetic O_2 evolution resulting from depriving the cultures of sulfate. Although technically successful, the sulfur-deprivation process is currently too expensive. In order to address the cost issue, we describe here the development of a continuous system that decreases the cost of H_2 production.

Approach

Subtask 1 (Hydrogenase Function and Molecular Engineering): There are a number of mutagenesis approaches to molecular-engineer enzymes for altered catalytic activity. Some of these approaches involve random changes in the enzyme's gene

sequence (either by *in vitro* error-prone polymerase chain reaction or gene shuffling), followed by high-throughput screening for the desired mutants. However, it is also possible to alter an enzyme's activity by applying a knowledge-based approach. In attempting to confer O_2 tolerance to algal hydrogenases, we chose the latter. Our approach is based on the hypothesis that inactivation of a hydrogenase depends on the access of O_2 to the enzyme's catalytic site. We assumed that this access occurred through a gas channel, a hydrophobic pathway identified in other hydrogenases by X-ray structure analyses (Montet et al., 1997; Nicolet et al., 1998) and identified by our group in algal [Fe]-hydrogenases (King et al., patent pending). According to a hypothesis presented for NiFe hydrogenases (Bernhard et al., 2001; Volbeda et al., 2002), a narrower channel might discriminate between the reaction product, H_2, and the inhibitor, O_2. We designed mutants in which residues with bulkier side chains are substituted for some of the small residues that line the gas channel. We report here the generation of the first site-directed hydrogenase mutant specifically designed to demonstrate increased tolerance to O_2 exposure. Future work will involve the generation of mutants with multiple amino acid substitutions in the gas channel, and it may also address the complementary use of random mutagenesis to target protein sequences that we cannot rationally relate to O_2 tolerance.

Subtask 2 (Biochemical and Process Characterization): The use of sulfur deprivation to metabolically shift the algal cultures from an O_2- to a H_2-production process was reported by UC Berkeley and NREL in 2000 (Melis et al., 2000). As originally conceived, the method involved cycles of O_2 production (in sulfur-replete medium) followed by cycles of H_2 production (in sulfur-deprived medium) [Ghirardi et al., 2000]. This temporal-separation batch process was studied by NREL and found to be too costly (Amos, 2000). Among the variables responsible for the high cost were the use of centrifugation to cycle the cultures from one phase to the next and the limited time during which the cultures photoproduce H_2. We investigated two approaches to remove the centrifugation step: (a) the use of judicious dilution of the sulfur-replete cultures to deprive them of sulfate (Laurinavichene et al., 2002) and (b) the design of a new two-photobioreactor system, based on cell growth under a sulfate chemostat in one reactor, followed by H_2-production under sulfur-deprived conditions in a second reactor. The cultures in the second bioreactor should produce H_2 continuously in the light but at limited rates.

Results

Subtask 1 (Hydrogenase Function and Molecular Engineering): The putative amino acid sequences of the two cloned algal hydrogenases, HydA1 and HydA2, were derived from their gene sequences. The amino acid sequences were then used to generate homology models of the respective protein structures, using the Swiss-Model program and further refinements by GROMOS (Forestier et al., 2003; King et al., submitted). Modeling was based on the known X-ray structure of another [Fe]-hydrogenase, CpI, found in the anaerobic bacterium *Clostridium pasteurianum* (Peters et al., 1998). Figure 1 shows the resulting structures of CpI, HydA1, and HydA2. The core regions of HydA1 and HydA2 exhibit a very high degree of structural similarity between each other and CpI, and the locations of the H-cluster catalytic sites overlap closely with those from CpI. The catalytic site consists of a 2Fe2S center linked by a cysteine residue to a 4Fe4S center, which is bound to the protein structure by three other cysteine residues (Peters et al., 1998; Nicolet et al., 1998). Moreover, the 2Fe2S center was shown to be ligated by unusual molecules such as CO, CN, and di(thiomethyl)amine (Nicolet et al., 2002). The main differences between the bacterial and the algal hydrogenases are (a) the presence of extra sequences in the N-terminal portion of CpI that bind additional FeS centers (Happe et al., 2002; Forestier et al., 2003), which serve as an electron relay from a soluble donor bound to the surface of the protein to the catalytic site, and (b) the presence of loops L1 and L2 in HydA1 and HydA2 that correspond to intervening gene sequences present only in the algal hydrogenases. Speculation suggested that the loops could be involved in the docking of ferredoxin (Winkler et al., 2002). We propose that their function could be related to regulating catalysis by transferring regulatory signals from the surrounding medium to the catalytic site.

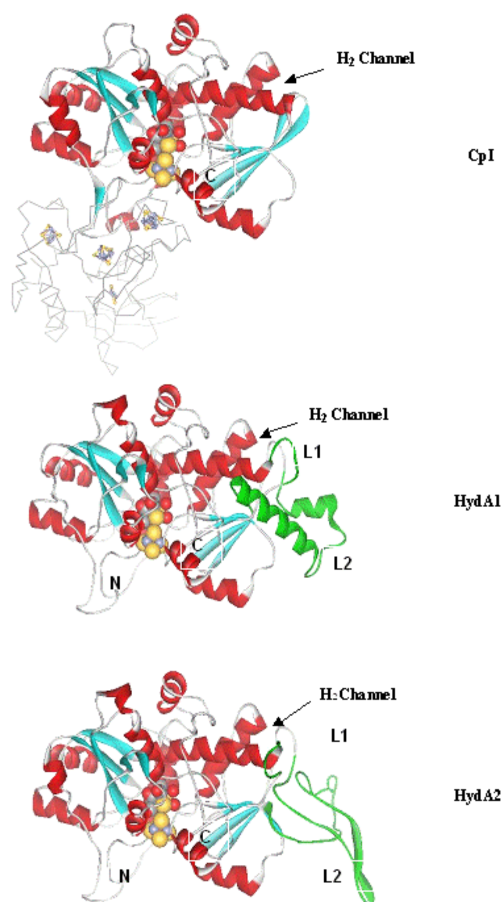

Figure 1. Model of the X-ray Structure of the *Clostridium pasteurianum* CpI and Homology Models of the Two Algal Hydrogenases, HydA1 and HydA2

The catalytic H-clusters in the three proteins are located in close proximity to a solvent-accessible area, termed the gas channel. Small, hydrophobic residues were identified as components of the channel wall. Homology modeling done at NREL suggested that mutating amino acid residues along the gas channel could narrow the channel in the region next to the catalytic site and in turn lower the access of O_2 (King et al., submitted). One of those residues, valine 240, was replaced by molecular engineering techniques with a bulkier counterpart, tryptophan. Table 1 shows that the H_2-production activity of the mutant (measured by two different assay methods, see legend) is more resistant to O_2 inactivation than the wild-type strain. This result confirms our initial hypothesis and justifies future molecular engineering techniques to further improve O_2 tolerance by modifying gas-channel residues.

Table 1. Effect of O_2 exposure on the rates of H_2 photoproduction by the WT and V240W strains of *Chlamydomonas reinhardtii*. Assay I measures the initial rate of light-induced H_2 production measured with a Clark electrode; assay II measures the rate of H_2 production induced by the addition of dithionite-reduced methyl viologen and is measured over a period of 10-15 min. The samples were exposed to the indicated concentrations of O_2 for 2 min preceding each assay.

O_2 concen-tration, %	% Uninhibited H_2-production rate			
	Assay I (light)		Assay II (MV_{red})	
	WT	V240W	WT	V240W
0	100	100	100	100
0.1	42	-	-	-
1	12	100	19	88
1.5		48	-	-
2	0	12	2.4	45
3	0	5		-
4	-	-	1.5	24

Subtask 2 (Biochemical and Process Characterization): Sulfur deprivation of algal cultures selectively inactivates photosynthetic O_2 evolution and creates an anaerobic environment. Anaerobic conditions are sufficient to induce the hydrogenase gene, initiate synthesis of the hydrogenase protein, and promote subsequent H_2 photoproduction. The latter cannot be sustained for more than 3-4 days, however, due to pleiotropic effects of sulfate deprivation on the algae at later times. We conducted a detailed study of the relationship among the different metabolic pathways under sulfur-deprived conditions and concluded that the initial rate of H_2 photoproduction is limited by the rate of reductant utilization. This results in an over-reduced plastoquinone pool (Antal et al., 2001 and in preparation). The limitation is not due to low hydrogenase activity (Ghirardi et al, submitted), but is possibly related to regulatory mechanisms associated with the non-dissipation of a proton

gradient, as suggested for anaerobic, sulfur-replete cultures (Lee and Greenbaum, 2002).

Since the initial rate of H_2 photoproduction cannot be increased easily by biochemical or engineering manipulations (Kosourov et al., 2003), we attempted to develop a system in which the cultures produce H_2 at a similar fixed rate but for much longer periods of time. Figure 2 shows the concept. In the first photobioreactor, the algal cells are grown in a chemostat mode (by continuous dilution with medium containing sufficient amounts of sulfate). The cultures in the second photobioreactor are initially sulfur-deprived and allowed to start photoproducing H_2. At that point, the second photobioreactor is connected to the first, and the H_2-producing cultures are continuously replaced by photosynthetically-competent cells from the first photobioreactor. The two main objectives of the above design are to continuously (a) replace sulfur-deprived cells with fresh cultures to avoid the long-term inhibitory effects of sulfur deprivation on H_2 photoproduction and (b) remove accumulated fermentation products from the H_2-photoproduction reactor.

The performance of the two-photobioreactor system was tested over a period of three weeks to meet our milestone for FY 2003. Figure 3 demonstrates that the system works as expected and is able to photoproduce H_2 at a rate that is constant during the first two weeks but decreases during the third week. Preliminary economic analyses indicate that the expected cost of the system is almost four times lower than that of the corresponding batch system (from $760 to $200/kg [Amos, unpublished]). Further improvements both to the system and to the organism by NREL, UC Berkeley and ORNL will be crucial to continuing to lower the cost of H_2 photoproduction.

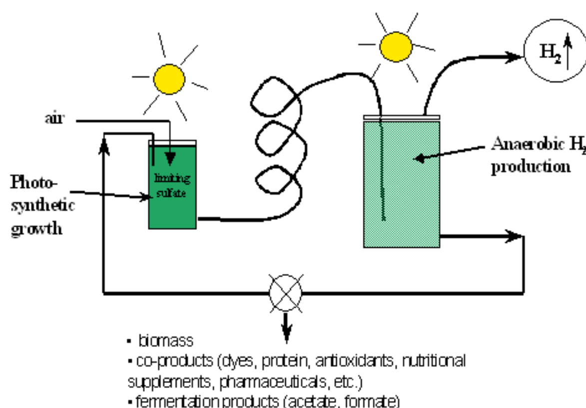

Figure 2. Schematic Diagram of the Two-Photobioreactor, Continuous Algal H_2 Photoproduction System

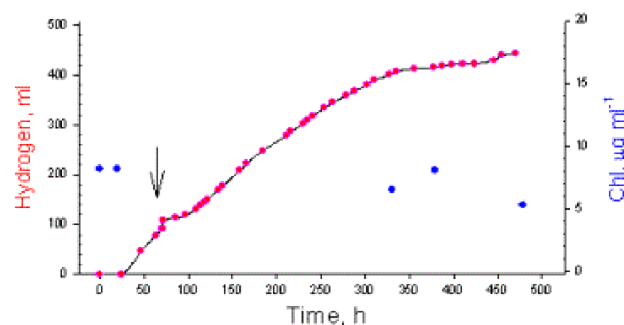

Figure 3. Accumulation of H_2 by illuminated, sulfur-deprived algal cultures in the continuous mode. The arrow indicates the time point at which the second photobioreactor was connected to the first. Notice that the cell density, indicated by the Chl concentration, decreased towards the end of the experiment.

Conclusions

- The generation of the first successful algal hydrogenase mutant (V240W) validates our knowledge-based mutagenesis approach to improve the O_2 tolerance of the enzyme and justifies our hypothesis that direct O_2 access to the catalytic site both inactivates enzyme activity and limits H_2 photoproduction.
- Algal H_2 photoproduction can be extended for much longer periods of time using the sulfur-deprivation method to physically (rather than temporally) separate O_2 and H_2 production activities; this approach currently lowers the cost of the process by about a factor of four when compared to the batch system.

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FY 2003 Publications

1. Laurinavichene, T., Tolstygina, I.V., Galiulina, R.R., Ghirardi, M.L., Seibert, M. and Tsygankov, A. (2002) Dilution methods to deprive *Chlamydomonas reinhardtii* cultures of sulfur for subsequent hydrogen photoproduction. Int. J. Hydrogen Energy 27, 1245-1249.

2. Flynn, T., Ghirardi, M.L. and Seibert, M. (2002) Accumulation of O_2-tolerant phenotypes in H_2-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection. Int. J. Hydrogen Energy 27, 1421-1430.

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5. Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L. and Seibert, M. (2003) Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. Eur. J. Biochem. 270, 2750-2758.

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7. Hahn, J.J., Ghirardi, M.L. and Jacoby, W.A. The effect of process variables on photosynthetic algal hydrogen production. Submitted.

8. King, P., Forestier, M., Zhang, L., Ghirardi, M.L. and Seibert, M. Molecular engineering of a *Chlamydomonas reinhardtii* hydrogenase for increased O_2 tolerance. Proceedings of the Boden Conference in Artificial Photosynthesis, Sydney, Australia. Submitted.

9. Ghirardi, M.L., Kosourov, S. and Seibert, M. Artificial photosynthetic systems for algal hydrogen production. Proceedings of the Boden Conference in Artificial Photosynthesis, Sydney, Australia. Submitted.

FY 2003 Presentations/Posters

1. Invited presentation at Oak Ridge National Laboratory, October 2002. “Photosystem II, the Engine for Algal Hydrogen Production”, M. Seibert.

2. Invited Mechanical Engineering Departmental Seminar, University of Colorado, Boulder, November 2002. “Algal H_2 Production—Physiology, Molecular Genetics and Process Development”, P. King, S. Kosourov, M.L. Ghirardi and M. Seibert (MS presented).

3. Invited presentation at the Boden Conference on Artificial Photosynthesis, Sydney, Australia, January 2003. “Modifying Photosynthesis for Continuous Algal Hydrogen Photoproduction”, M.L. Ghirardi, S. Kosourov and M. Seibert (MLG presented).

4. Invited presentation at the Boden Conference on Artificial Photosynthesis, Sydney, Australia, January 2003. “Cloning and Molecular Engineering of *Chlamydomonas* [Fe]-hydrogenases for Oxygen Tolerance”, M. Seibert, P. King, L. Zhang, M. Forestier, S. Schwartz, T. Happe and M.L. Ghirardi (MS presented).

5. Invited seminar in the Research School of Biological Sciences, Australian National University, January 17, 2003. “Algal H_2 Production—Physiology, Process Development, and Molecular Genetics”, P. King, S. Kosourov, M.L. Ghirardi and M. Seibert (MS presented).

6. Presentation at the University of Colorado’s Super Microbiology Group, April 2003. “Cloning of a Second Putative [Fe]-Hydrogenase from the Green Alga *Chlamydomonas reinhardti*i”, M. Forestier, P. King, L. Zhang, M. Posewitz, S. Plummer, S. Smolinski, M. Ghirardi and M. Seibert (MLG presented).

7. Invited presentation at the U.S. Air Force AFOSR workshop on Biohydrogen and Artificial Photosynthesis, Denver, CO, April 2003. “Algal Hydrogen Photoproduction”, M.L. Ghirardi.

8. Poster at the Symposium on the Biotechnology for Fuels and Chemicals, Breckenridge, CO, May 2003. “Algal Hydrogen Production – Physiology and Process Development”, M. Seibert, L. Zhang, V. Makarova and M.L. Ghirardi (MS presented).

9. Invited presentation at the Bio2003 conference in Washington D.C., June 2003. “Algal Hydrogen Photoproduction”, M.L. Ghirardi, A. Fedorov, P. King, S. Kosourov, M. Posewitz, S. Smolinski, L. Zhang and M. Seibert (MLG presented).

Patents

1. Kosourov, S., Ghirardi, M. and Seibert, M. "Multi-stage microbial system for continuous hydrogen production". Patent application filed October 2002.

2. King, P., Ghirardi, M.L. and Seibert, M. "Oxygen-tolerant hydrogenases and methods for designing and making same". Provisional patent application filed April 18, 2003.

FY 2003 Videos

1. City in a Pyramid, Discovery Channel, April 2003

2. Video for KLTV Channel 8, Lakewood, CO, March 2003