II.K.4 Regulation of H_2 and CO_2 Metabolism: Factors Involved in Partitioning of Photosynthetic Reductant in Green Algae

Maria L. Ghirardi (Primary Contact), Mike Seibert, Matthew Posewitz and Matthew Wecker
National Renewable Energy Laboratory
1617 Cole Blvd.
Golden, CO 80401
Phone: (303) 386-6312, Fax: (303) 384-6150
E-mail: maria_ghirardi@nrel.gov

DOE Program Officer: Richard Greene
Phone: (301) 903-6190
E-mail: Richard.Greene@science.doe.gov

Subcontractor:
Matthew Posewitz, Colorado School of Mines, Golden, CO 80401

Objectives

The primary objective of this research effort is to develop fundamental understanding about the partitioning of photosynthetic reductant at the level of ferredoxin between the H_2-producing, hydrogenase pathways (active only under anaerobic conditions) and the CO_2-fixing, Calvin-Benson cycle. Specific objectives include: (a) identify protein factors whose expression may be required for optimal hydrogenase expression and activity; (b) identify the promoter regions and transcriptional elements activating the expression of hjydrogenase; and (c) determine how C. reinhardtii senses O2 and transduces this signal into gene regulation.

Technical Barriers

Besides the sensitivity of the hydrogenase to O_2, one of the other major barriers to efficient photobiological H_2 production by photosynthetic organisms is the existence of competing metabolic pathways for photosynthetic reductants, such as CO_2 fixation. In order to attain the theoretical maximum light conversion efficiency of 10% from sunlight to H_2, it is essential that most of the photosynthetic reductant be utilized for H_2 production, while concomitantly allowing the cells to store some energy in the form of starch to run their internal metabolism. Once the factors that are involved in this partitioning are known, we expect to learn how to modulate them to achieve efficient H_2 photoproduction and basal cell metabolism occurring simultaneously.

Abstract

The objective of this proposed research is to continue to develop fundamental understanding about the regulation of partitioning of photosynthetic reductants (at the level of ferredoxin) between the H_2-production and the CO_2-fixation pathways. Our analysis of *C. reinhardtii* mutant libraries in the previous funding periods for strains having attenuated H_2 production has led to the discovery of maturation proteins that are required for the proper assembly of hydrogenases, and to the discovery of the STA7 isoamylase and of the putative 3-hydroxybutyrate dehydrogenase genes, reflecting the importance of starch and lipid metabolic pathways for H_2 production. We have recently shown the presence of heme-binding, FixL-like proteins in *C. reinhardtii*. Such proteins likely play a role in the O_2-sensing mechanism and may mediate components of the anoxic regulatory response. The proposed work will continue to (a) determine what other protein factors may be required for optimal hydrogenase expression and activity, (b) identify O_2-sensor proteins in *C. reinhardtii* that regulate the expression of the reversible hydrogenase, (c) conduct analyses of the promoter regions for *HYDA1* and *HYDA2* genes, encoding the two algal hydrogenases; and (d) understand the simultaneous or complementary regulation of the competitive CO_2 fixation pathway under anaerobic conditions.

Progress Report

We have screened through a *C. reinhardtii* mutagenesis library (6,000 transformants) for colonies deficient in H_2-production capability. A bank of eleven distinctly different *C. reinhardtii* mutants were isolated for having attenuated H_2 production, while maintaining wild-type (WT) rates of photosynthetic O_2 evolution and respiration. Among these eleven mutants, two mutants have been fully characterized. One mutant contained a disrupted isoamylase gene (*STA7*) and its characteristics were described in detail previously. The other mutant that we characterized contained a disrupted novel [FeFe]-hydrogenase assembly protein gene, *HYDEF*, and has also been previously reported. The flanking DNA for three additional mutants has been obtained and the disrupted genes appear to include: 1) a putative 3-hydroxybutyrate dehydrogenase (*3-HBD)*; 2) a potential quinone oxidoreductase; and, 3) a disruption neighboring a potential transcriptional regulator, ferredoxin, or kinase. A mutation in 3-HBD indicates that lipid metabolism is involved in H_2 production, a finding that could correlate with chloroplast membrane lipid degradation upon sulfur deprivation and ensuing H_2 production in *C. reinhardtii* [64]. This mutant has attenuated hydrogen production in early to mid exponential growth phase, but normal H_2 production in older cultures. PCR and reverse transcription PCR assays indicate a duplication of the *3-HBD* gene during insertion mutagenesis. One of the duplicated genes is disrupted by the insert, while the other gene appears to be intact, with mRNA for *3-HBD* at approximately wild-type levels. This mutant is currently being back-crossed by Patrice Hamel (Ohio State University) against a WT cell line prior to further analysis. An additional library of insertion *ApaH* paromycin mutants is being created in order to streamline some of the difficulties encountered in the original library. These experiments will help to further define the biochemical pathways required for H_2 production in *C. reinhardtii*.

As a second approach to finding other regulatory genes, *C. reinhardtii* proteins with homology to O_2-sensing proteins were identified by our group. Analysis of the *Chlamydomonas reinhardtii* genome indicates a number of closely-related homologs to eubacterial FixL proteins. Nine of these Fi<u>x</u>L <u>l</u>ike homologs (FXL) have a core PAS domain that is homologous to the FixL O_2-sensing PAS domains of the nitrogen-fixing bacteria *Bradyrhizobium japonicum, Sinorhizobium meliloti* and *Rhizobium leguminosarum*. Each of the putative homologs has multiple transmembrane-spanning domains, which are typical of the bacterial FixL homologs. Additional homology includes conserved α-helical loops, β-sheet structures, and critical residues required for binding a heme moiety transmitting the presence of heme-bound O_2 to an autophosophorylation site of a conserved histidine residue within a neighboring histidine kinase domain. However, the *Chlamydomonas* FixL homologs do not have the typical histidine autophosphorylation site. Two of the *Chlamydomonas* FixL heme-binding domains were cloned as truncated genes (*FXL1PAS* and *FXL5PAS*) and the corresponding expressed proteins in *E. coli* were shown to coordinate heme. The heme moiety is visible on an *o*-dianisidine-stained, SDS-PAGE denaturing gel, as either free heme or bound to the truncated FXL proteins. Purified cell extracts of the background strain of *E. coli* harboring only the vector plasmid did not heme-stain prominently when the cells were grown under identical conditions, nor do truncated FXL proteins stain when purified under denaturing conditions, although upon reconstitution with 100 µM hemin, the heme group again migrated with the protein band. The spectral properties of FXL1PAS and FXL5PAS reveal absorbance peaks at 415 nm and 530/560 nm that are the characteristic bands of protein-bound heme and similar to that of oxy-*Rm*FixL, although typically the peak at 560 nm is more intense than that at 530 nm. Upon reduction with 10 mM sodium dithionite, the primary 415 nm Soret bands of both FXL1PAS and FXL5PAS shift about 15 nm downfield, and the ratio of the absorbance of the 560 nm band relative to the 530 nm band increases, as is typical of heme-binding proteins. Preliminary characterization of the FXL1 and FXL5 heme-binding domains indicates that these proteins are able to coordinate heme. We are currently testing the ligand binding characteristics of these domains to determine the effects of O_2 coordination. These data will be correlated with the transcriptional level of the FixL homologs resulting from exposure to O_2 and other gases during culture. Using microarray analysis, two of the FixL homologs (FXL1 and FXL2) were observed to have increased transcript levels upon dark, anerobic adaptation. To date there have been no other reports of O_2-sensing regulatory proteins in *Chlamydomonas*. Analysis of these FixL homologous genes may prove invaluable for understanding the regulation of fermentative metabolism and the production of H_2 under conditions of low O_2 tension.

Future Directions

- Identify additional protein factors whose expression may be required for optimal hydrogenase expression and activity;
- Identify the promoter regions and transcriptional elements activating the expression of hydrogenase in *C. reinhardtii*;
- Determine whether the FXL proteins sense O_2, and establish how the signal for O_2 is transmitted from the sensing proteins to cellular regulation. In particular, determine whether these sensing proteins relate to hydrogenase gene expression and H_2 production in *Chlamydomonas reinhardtii*.

Publications (including patents) acknowledging the grant or contract in 2007:

1. Ghirardi, ML, MC Posewitz, PC Maness, A Dubini, J Yu and M Seibert. **2007**. Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. *Ann. Rev. Plant Biol.* 58, 71-91.

2. Nagy, LE, JE Meuser, S Plummer, M Seibert, ML Ghirardi, PW King, D Ahmann and MC Posewitz. **2007**. Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries. *Biotechnol. Lett.* 29: 431-430.