Molecular Engineering of Algal H_2 Production

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Abstract

Hydrogen production by green algae has significant advantages over other photobiological systems: ATP production is not required, high theoretical efficiencies are possible, and water is used directly as the source of reductant without the need to produce biomass or to store intermediary carbon metabolites. However, algal H_2 photoproduction is sensitive to O_2, a co-product of photosynthesis, and this sensitivity is a major factor currently limiting the use of algal systems for H_2 production. We are investigating ways to surmount this problem, by focusing on the amino acid sequence and structure of the reversible [Fe]-hydrogenase (the enzyme that releases H_2 gas). Previous studies (McTavish *et al*., 1995; Ghirardi *et al.*, 1997; Seibert *et al.*, 2001; Flynn *et al.*, 1999; 2002) indicate that mutagenesis can be used to decrease the O_2 sensitivity of the hydrogenase and thus eventually lead to a system that produces H_2 under aerobic conditions.

Last year, we cloned and sequenced the *hydA1* [Fe]-hydrogenase gene in *Chlamydomonas reinhardtii*. We also cloned and partially sequenced a second, unexpected hydrogenase gene, *hydA2.* This year, we completed the sequencing of *hydA2*, confirmed that it is expressed at the mRNA level and is a different gene than *hydA1*, and started to characterize both genes. We found that the *hydA2* gene has a similar degree of homology to other [Fe]-hydrogenases as *hydA1*, and it contains all the motifs characteristic of these hydrogenases. We also found that *hydA2* is a nuclear gene that is located on a different chromosome from *hydA1.* The two corresponding transcripts, *hydA1* and *hydA2* are induced almost equally under photoheterotrophic and photoautotrophic anaerobic conditions, and are equally inactivated by exposure to O_2. In order to investigate differences in the physiological role of the two cloned algal hydrogenases, we have raised antibodies specific against each of the two enzymes. These antibodies will be used for detailed protein expression studies in the future. Finally, we did some structural modeling on the purported amino acid sequences of HydA1 and HydA2 and have identified regions of the proteins to target for future mutagenesis in order to improve their O_2 tolerance.

Introduction

Hydrogen metabolism, catalyzed by [Fe]-hydrogenases in green algae, was first observed 60 years ago in *Scenedesmus obliquus* (Gaffron, 1939; Gaffron and Rubin, 1942). Since then, hydrogenase enzymes that either uptake or evolve H_2 have been found in many green algae (Weaver *et al.*, 1980), including *Chlamydomonas reinhardtii*. In this alga, H_2 reactions are catalyzed by a monomeric, 49-kDa, reversible [Fe]-hydrogenase enzyme, which has been isolated to purity by Happe and Naber (1993).

Other [Fe]-hydrogenases, identified in a small group of anaerobic microbes (bacteria, protists and other green algae), catalyze either H_2 production or H_2 uptake *in vivo* (Adams, 1990; Vignais *et al*., 2001). They play an important role in the anaerobic energy metabolism of these

organisms, mainly by reoxidizing accumulated reducing equivalents. All [Fe]-hydrogenases incorporate a [2Fe-2S] cluster bridged by a cysteine residue to a [4Fe-4S] cluster at the catalytic site (the H-cluster), and have unusual ligands such as CO, CN and di(thiol)methylamine (Fan and Hall, 2001; Nicolet *et al.*, 2001). Three other conserved cysteine residues bind the H-cluster to the protein matrix. Most of these enzymes also contain additional iron-sulfur centers that act as electron relays to and from donor and acceptor carriers (Peters, 1999; Nicolet *et al.*, 2000). However, these cofactors are absent from the green algal enzymes (Florin *et al.*, 2001; Forestier *et al.*, 2001; Wünschiers *et al*., 2001; Happe and Kaminski, 2002; Winkler *et al.*, 2002). In addition, [Fe]-hydrogenases usually exhibit high specific activity but are easily inactivated by either O_2 or CO. Oxygen inactivation is thought to occur by the direct interaction of O_2 with the [2Fe-2S] center on the catalytic H-cluster (Adams, 1990; Chen *et al.*, 2002).

We have cloned and sequenced two [Fe]-hydrogenase genes from *C. reinhardtii*, *hydA1* and *hydA2* (Forestier *et al.*, 2001; Forestier *et al.*, submitted), and deposited their sequences in Genbank (accession numbers AY055755 and AY055756). The *hydA1* gene cloned in our lab is 100% homologous to a *C. reinhardtii* hydrogenase gene cloned by L. Mets in 2000 and also reported by Happe and Kaminski (2002). The hydrogenase encoded by *hydA1* contains the 24 amino acid residues present in the N-terminal portion of the isolated *C. reinhardtii* reversible hydrogenase, and shows a high degree of homology to previously cloned [Fe]-hydrogenases from anaerobic bacteria. It was proposed that the *hydA1* gene encodes a functional hydrogenase (Happe and Kaminski, 2002). However, not much is known yet about the putative hydrogenase encoded by *hydA2.* Sequence analyses suggests that *hydA2* carries a signal sequence encoding for a chloroplast transit peptide of about 63 residues (Forestier *et al.*, submitted), which would indicate that its gene product is located in the chloroplast. Both *hydA1* and *hydA2* sequences display the three motifs found in the catalytic sites of other [Fe]-hydrogenases, motif 1 (PMFTSCCPxW), motif 2 (MPCxxKxxExxR) and motif 3 (FxExMACxGGCV) [Forestier *et al.*, 2001]. Finally, comparisons of *C. reinhardtii* (putative) HydA2 with the HydA1 [Fe]-hydrogenase from *C. reinhardtii* and the [Fe]-hydrogenase sequence from *S. obliquus* show 66% and 61% identity, respectively. It is clear that these results reflect the close phylogenetical relationship between all algal [Fe]-hydrogenases.

In this paper, we describe the initial characterization of the second hydrogenase gene in *C. reinhardtii*, *hydA2.* Our evidence shows that HydA1 and the putative HydA2 protein are encoded by different genes, *hydA2* is expressed, and its expression is regulated by anaerobiosis. Future studies will be done to differentiate between the roles of HydA1 and putative HydA2 in H_2 photoproduction under different physiological conditions. These studies will determine which of the enzymes should be the focus of our mutagenesis efforts to generate O_2-tolerant mutants. We also show that the modeled structures of HydA1 and HydA2 are very similar to each other and to that of the [Fe]-hydrogenase from the anaerobic bacterium, *Clostridium pasteurianum*, for which there is a solved X-ray crystal structure (Peters *et al.*, 1998). Careful analyses of the modeled structures of the two algal hydrogenases will also help to direct our future efforts in the generation of O_2-tolerant mutants for sustained H_2 production under aerobic conditions

Materials and Methods

Cell Growth and Anaerobic Induction

Chlamydomonas reinhardtii strain 400 (cell wall-less) was grown photoheterotrophically on TAP medium (Harris, 1989), supplemented with 5% CO_2 in air, or photoautotrophically on Basal Salts (BS) medium (Flynn *et al.*, 1999; Flynn *et al.*, 2002) under cool white fluorescent light (150 $\mu Em^{-2}s^{-1}$, PAR). Cells were harvested at an OD_{750} of about 1 (basically due to light scattering by the

sample) corresponding to a concentration of about 15 µg Chl/ml (obtained from a calibration curve), and the harvested cells were anaerobically induced as previously described (Ghirardi *et al*. 1997) in phosphate buffer for 4-5 h.

Southern and Northern Blot Analyses

Total genomic DNA was prepared from *C. reinhardtii*, using the Qiagen DNeasy Genomic Kit (Qiagen, Valencia, CA), and digested with *Pst*I. Digested DNA was separated by agarose gel electrophoresis and blotted onto a Nytran N+ nylon membrane for Southern blot analysis. Total RNA was isolated at different times after anaerobically-inducing the samples, using the SNAP RNA Isolation Kit (Invitrogen). DNA was removed by treatment with RNase-free DNaseI (0.013 units/µl). The isolated RNA was separated by electrophoresis and then blotted as above for Northern analyses. ^{32}P-radiolabeled probes specific for either *hydA1* or *hydA2* were generated and denatured probes were hybridized to the membranes overnight at 65°C. Following hybridization, the membranes were washed and exposed to X-ray film at –80°C for 1-4 days. Blots were scanned with a HP Scanjet 5300C optical scanner connected to a PC.

H_2-Evolution Assays

The assays were done in MOPS buffer (50 mM, pH 6.8) with two Clark electrodes (YSI 5331, Yellow Springs, OH) as described by Ghirardi *et al*. (1997). The O_2 concentration in the cuvette was set close to zero with Ar, and 200 µl of anaerobically induced cell suspension were injected into the buffer. The cell suspension was illuminated (incandescent light filtered through 1% $CuSO_4$; 320 µE m^{-2} s^{-1}, PAR) for three minutes, and the initial rates of H_2 production were estimated from the initial slopes of the curves.

SDS-PAGE and Western Blot Analysis

Control and anaerobically-induced cells were harvested by centrifugation at 3,000 x g for 3 min at 4°C. The cells were resuspended in solubilization buffer containing 0.5 M Tris-HCl (pH 6.8), 7% SDS, 20% glycerol, and 2 M urea. Chlorophyll (a + b) content of the samples was measured as 95% ethanol extracts by the method of Spreitzer (Harris, 1989). Subsequently, β-mercaptoethanol was added to the resuspended, solubilized cells at a final concentration of 10%. Protein electrophoresis was carried out by SDS-PAGE according to Laemmli (1970), using 4.5% acrylamide in the stacking gel and 12.5% acrylamide in the resolving gel. Unless otherwise stated, the gel lanes were loaded with an equal amount of chlorophyll (12 nmol). Electrophoretic transfer of the SDS-PAGE resolved polypeptides to nitrocellulose was followed by incubation with polyclonal antibodies designed specifically against HydA1 and HydA2. Western-blot analyses were carried out following the ECL™ Western blotting assay kit procedures (Amersham, Piscataway, NJ). The antigen-antibody complex was detected using a horseradish-peroxidase-linked goat anti-rabbit secondary antibody.

Homology Structure Modeling

Homology structural models of the HydA1 and the putative HydA2 hydrogenases to the known structure of the *Clostridium pasteurianum* CpI enzyme (Peters *et al.*, 1998) were generated using the Swiss-Model program (Guex and Peitsch, 1997). ClustalW alignments of the processed HydA1 peptide sequence and the predicted processed HydA2 sequence to CpI were used to manually optimize the threading alignments. Final versions of the model structures

were submitted to Swiss-Model for validation. Resulting homology structures were then further refined by energy minimization techniques using GROMOS.

Results and Discussion

Southern and Northern Analyses of *hydA1* and *hydA2*

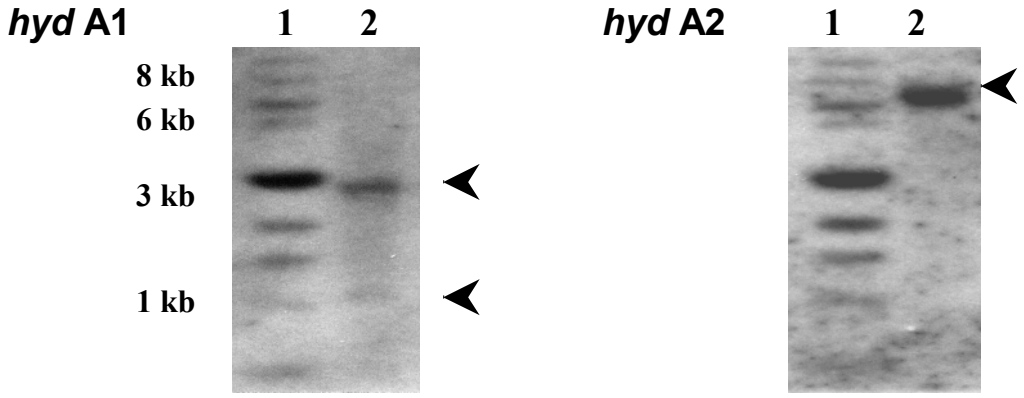
In order to determine whether HydA1 and HydA2 are encoded by distinct nuclear genes, *C. reinhardtii* genomic DNA was purified, digested with *Pst*I and probed separately with *hydA1*- and *hydA2*-specific DNA probes. Figure 1A shows that the *hydA1* probe, which overlaps a single *Pst*I restriction site of the *hydA1* genomic sequence, detected two *Pst*I restriction digest fragments of < 3 kbp and > 1 kbp, whereas the *hydA2* probe, which lacks the same *Pst*I restriction site, detected only a single fragment at > 6 kbp. This clearly demonstrates that *hydA1* and *hydA2* are located on separate *Pst*I fragments, and hence they represent different nuclear genes. The two hydrogenase sequences also map on different linkage groups, confirming conclusively that *hydA1* and *hydA2* are two different genes located on different chromosomes.

Exposure of *C. reinhardtii* to anaerobic conditions is accompanied by the induction of hydrogenase activity. Figure 1B shows a peak in induced H_2 photoproduction within 90 min of anaerobic treatment. The expression of the *hydA1* gene under anaerobic conditions is regulated at the transcriptional level (Happe and Kaminski, 2002). The expression of *hydA2* was studied and compared to that of *hydA1* by Northern blot analyses (Fig. 1B) under the same conditions used to induce activity. No detectable levels of either *hydA1* or *hydA2* transcripts were observed in TAP-grown cells at the time of harvest, but transcripts accumulated as a function of the length of the anaerobic treatment, reaching a maximum after about 90 min treatment and paralleling the induction of H_2-production activity. The induction of both *hydA1* and *hydA2* transcripts was found to be sensitive to oxygen, with transcript levels increasing rapidly as the cultures became anaerobic and decreasing to low but detectable levels upon a 15-min exposure to O_2 (not shown; Forestier *et al.*, submitted). When the same experiment was repeated with cells grown in photoautotrophic basal salts (BS) medium, H_2-production activity was induced more slowly, peaking at 240 min, but *hydA1* and *hydA2* transcript accumulation were also similar (not shown, but see Forestier *et al.*, submitted). The response of the transcript levels to O_2, in BS-grown cultures was more extreme: a 15-min exposure resulted in almost total loss of both *hydA1* and *hydA2* transcripts.

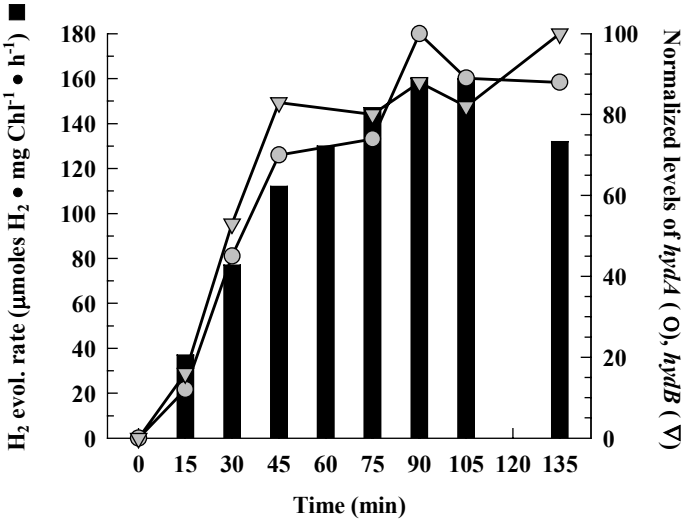
Generation of Specific Anti-HydA1 and Anti-HydA2 Antibodies

We designed two specific oligopeptides containing sequences that are specifically found in either HydA1 (15 residues) or purported HydA2 (14 residues). A third oligopeptide of 15 residues was also designed, and it was composed of sequences common to both algal enzymes (but not common to other non-algal [Fe]-hydrogenases). The three oligopeptides were synthesized by Sigma Genosys (The Woodlands, Texas), coupled to a carrier protein, KLH, and injected into rabbits. The resulting sera were tested in our laboratory and yielded three separate antibody preparations, all of which recognized a protein band at about 47 kDa. They will be used to uniquely identify the two different algal proteins.

A



B



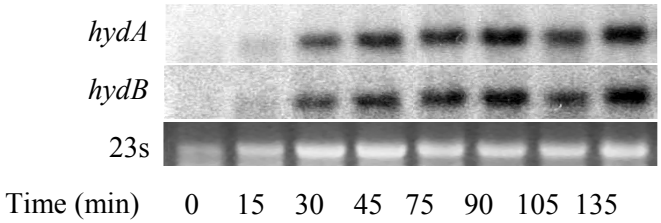


Figure 1. (A) Southern blot analyses of *PstI*-digested *C. reinhardtii* DNA probed with, respectively, *hydA1*- or *hydA2*-specific DNA probes. The arrows indicate the location of the restriction digest fragments. **(B)** Results from Northern blot analyses of RNA isolated from cultures anaerobically-induced for different periods of time and probed with *hydA1*- or *hydA2*-specific probes. The bars represent H_2-production activity measured concomitantly. Gels at the bottom show the original Northern blot data.

Characterization of the specific antibody reaction to HydA1 and HydA2 was done by over-expressing the two proteins in *E. coli* (using the pET-16b system, Novagen, Madison, WI) and testing cell extracts against the previously-generated antibodies. Figure 2A shows a Coomassie-blue stained SDS-PAGE gel of cell extracts from two *E. coli* clones that express, respectively, either HydA1 or HydA2. Both over-expressed HydA1 or HydA2 can be seen at about 47 kDa in their respective gels. Figure 2B demonstrates that, as expected, the anti-HydA2 antibody shows very little cross-reaction with the HydA1 protein. Finally, the two antibodies are being tested against cell extracts from *C. reinhardtii*.

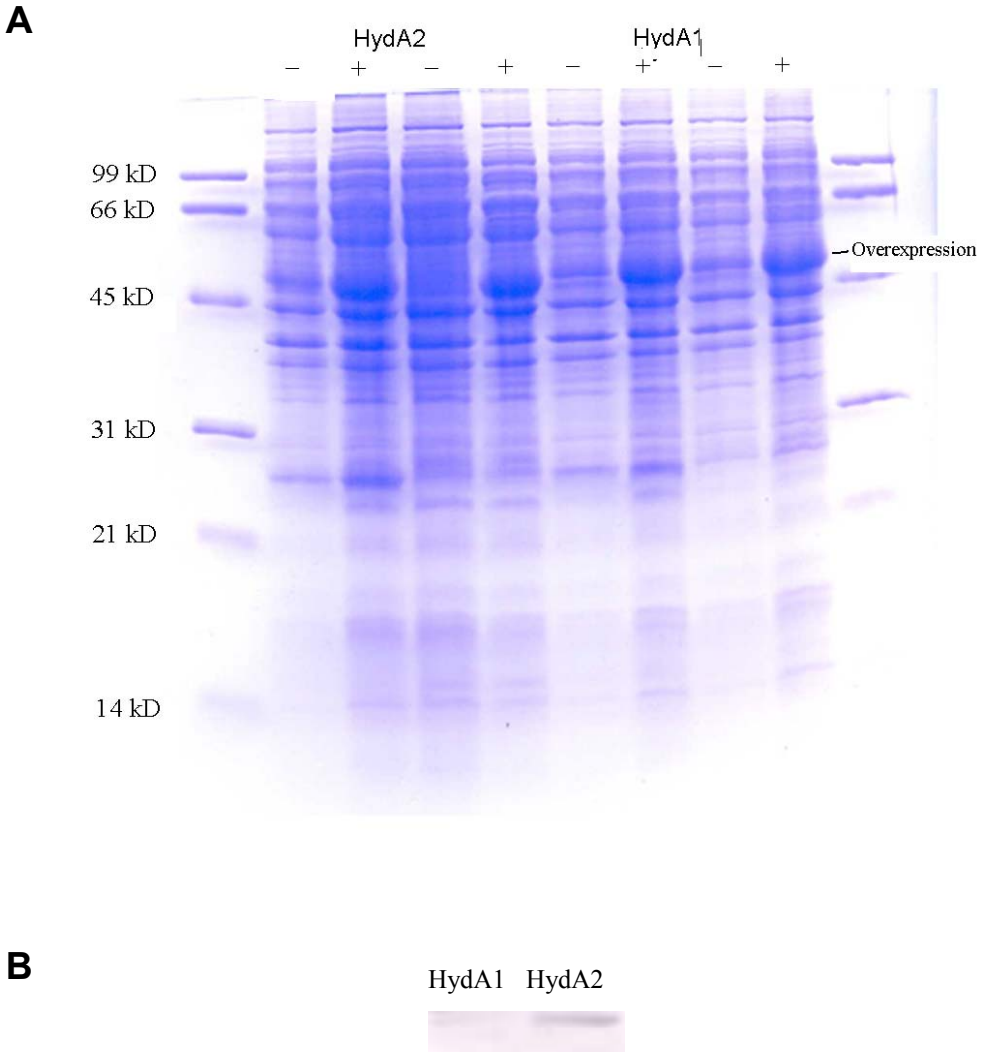


Figure 2. (A) SDS-PAGE of the cell extracts from *E. coli* over-expressing either the *C. reinhardtii hydA1* or *hydA2* gene. The lanes marked "–" represent control samples. **(B)** Western blots of *E. coli* cell extracts over-expressing either HydA1 or HydA2, probed with the HydA2-specific antibody.

Homology Modeling

In the absence of the actual purified, native proteins and X-ray crystal data, the predicted structures of HydA1 and of the putative HydA2 hydrogenase were generated by homology modeling of the processed peptide sequences against the known structure of the CpI [Fe]-hydrogenase, as described in the Materials and Methods. The core regions of HydA1 and HydA2 exhibit a very high degree of structural similarity between each other and with CpI (Forestier *et al.*, submitted). Fig. 3 shows a surface view into HydA1 down the "gas channel" thought to be the pathway that allows H_2 to diffuse out of the interior of the protein to the surface. The red dot at the center of the figure shows an exposed interior Fe atom on the [2Fe2S] center of the H-cluster, where H_2 gas is probably released following catalysis. Mostly hydrophobic residues form the surface of the channel, whose diameter is also wide enough to allow O_2 molecules to access the catalytic site. Similar channels have been described in bacterial [NiFe]-hydrogenases, and there is now evidence in the literature suggesting that narrowing of this channel in bacterial [NiFe]-hydrogenase sensor proteins is the basis for greatly improved O_2 tolerance in these proteins (Volbeda *et al.*, 2002). Mutagenesis of the residues along the channel might prevent O_2 from interacting with the H-cluster.

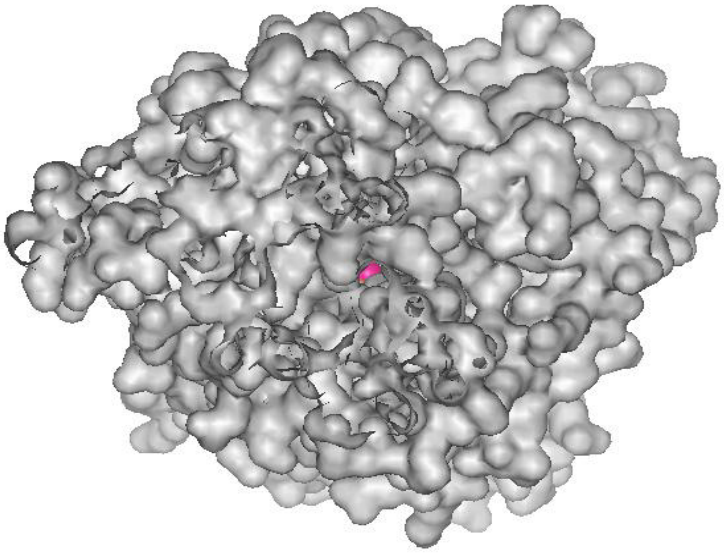


Figure 3. Surface model of HydA1. The red dot in the center of the structure represents the [2Fe2S] center of the H-cluster.

Previous studies of the HydA1 peptide sequence (Melis and Happe, 2001) and examination of the HydA2 peptide sequence alignments have identified unique insert regions that correspond to common features of all algal [Fe]-hydrogenases but are not found in the sequences of non-algal [Fe]-hydrogenases. One such region of eight amino acids is located near the N-terminus. The second unique region consists of 45 (HydA1) and 54 (HydA2) amino acids, respectively, and is located near the C-terminus. Although the extra regions map to different ends of the peptide sequences, the structural models predict them to be located on the same face of the proteins as surface-exposed loops. Both loops are connected to the H-cluster via either a α-helix or a β-sheet. We suggest that this arrangement could provide the means to transduce signals between the stroma and the catalytic center of the enzyme, and mutagenesis of its

component residues could perhaps lead to alterations in the regulation of the enzyme activity. This could be a second target for mutagenesis.

In [Fe]-hydrogenases from different sources (bacterial, archae, protist), electron transfer to the catalytic center occurs by means of accessory ferredoxin-like FeS centers that are bound to cysteine residues in the hydrogenase structure. Algal hydrogenases lack these electron relays (Melis and Happe, 2001) and electrons are donated directly to the H-cluster active site from soluble ferredoxin. The H-cluster in CpI is in close proximity to a solvent-accessible cavity (Nicolet *et al.*, 1999). The corresponding cavity region in the algal hydrogenases corresponds to a large (~13-16-angstrom diameter) solvent-exposed pocket. The existence of this pocket is due to the lack of a CpI-like N-terminal region in HydA1 and HydA2. The H-cluster [4Fe-4S] is located near the center of this pocket, and it is partially exposed to the solvent. However, the [4Fe-4S] cluster is protected by hydrophobic amino acid residues. Our structural models of HydA1 and HydA2 have been used to identify a potential ferredoxin-docking surface, which is lined with positively-charged residues (not shown) that complement the surface shape and charge structure of the corresponding algal ferredoxin. The binding affinity of the hydrogenase to ferredoxin, 10 μM (Roessler and Lien, 1982), could probably be affected by mutagenesis of residues on this surface with the goal of increasing the electron transfer from ferredoxin to the hydrogenase.

The availability in our laboratory of cloned genes and structural modeling capability will allow us to start mutagenesis attempts to increase the O_2 tolerance of the two algal enzymes. Our approach will involve both random error-prone PCR mutagenesis techniques and site-directed mutagenesis of specific amino acid residues (knowledge-based mutagenesis). Desirable mutants will be detected with the chemochromic-screening technique that we have used in the past to identify O_2-tolerant chemically-induced mutants (Seibert *et al.*, 2001). These mutants would then be tested for their capacity to produce H_2 under anaerobic conditions and hopefully used in future applied systems.

Acknowledgements

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