

Direct Solar Biohydrogen

Investigator: James Swartz, Professor, Chemical Engineering and Bioengineering, Stanford University

Sponsor: Global Climate and Energy Project

Objective: The quantitative objective of this project is to demonstrate photobiological conversion of solar energy into molecular hydrogen at an efficiency of 7% for a 2-hour duration. The organism that performs this conversion will use a shuttle protein, ferredoxin, to transfer electrons from the reaction of water photolysis to an evolved, oxygen-tolerant hydrogenase enzyme.

Background: As solar energy is captured by an organism's photolysis center (photosystems I and II), water is split into protons, electrons, and molecular oxygen. In many organisms, the electrons are transferred directly to the electron carrying protein, ferredoxin. The aim of this project is to engineer an organism in which a hydrogenase enzyme accepts those electrons from ferredoxin in order to generate hydrogen. However, the side product of photolysis, oxygen, inactivates all known hydrogenases. Thus, the first and most significant challenge for this project is to evolve a natural hydrogenase enzyme to become oxygen tolerant.

Hydrogenases are very complicated enzymes. There are two general types, the Ni-Fe and Fe-only hydrogenases. The group has focused on the Fe-only enzymes as they provide much faster catalytic rates for hydrogen production. The 3-D structure has been determined for the CpI hydrogenase from *Clostridium pasteurianum* as shown in Figure 1. The enzyme has five Fe-S centers, and the catalytic active site is a complicated 6Fe-6S center stabilized by carbon monoxide and cyanide ligands. This oxygen-sensitive catalytic center is buried in the center of the hydrogenase. The group's working hypothesis is that the protein structure can be modified to exclude oxygen from the active site while still allowing protons to enter and hydrogen to exit. To evolve this or a similar hydrogenase to become oxygen tolerant, they will use a process called "protein evolution".

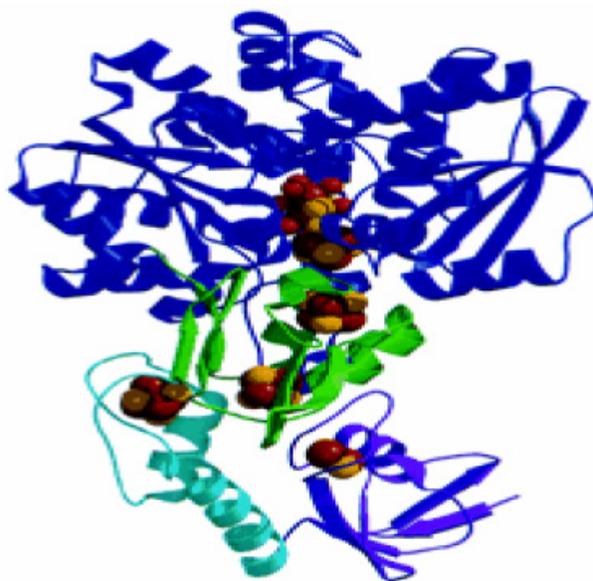


Figure 1: 3-D structure of the Fe-hydrogenase from *Clostridium pasteurianum*.

Approach: Protein evolution can be a long and difficult endeavor. It is important to be able to quickly express and evaluate the hundreds of thousands of candidates that will

need to be tested. They will use cell-free protein expression to produce the hydrogenase candidates directly from the products of polymerase chain reactions (PCR). In the group's previously funded work, they successfully produced a simpler iron sulfur protein with the cell-free system and then progressed to the expression of active hydrogenases.

Flynn *et al.* have shown that an *in vivo* mutagenesis and selection approach resulted in a 10-fold increase in oxygen tolerance for the *Chlamydomonas reinhardtii* hydrogenase. Although the resultant enzyme was still much too oxygen-sensitive to be useful, the result shows that oxygen tolerance can be evolved while retaining hydrogenase activity. The cell-free evolution approach is a much more powerful and focused approach and is expected to evolve oxygen tolerance more rapidly and more completely.

In order for *Synechocystis*, the photosynthetic organism to be engineered for hydrogen production (Koksharova *et al.*), to produce hydrogen at the targeted rates, several steps must be taken. The hydrogenase must be expressed and activated in the organism, the ferredoxin must be over-expressed, and an uncoupler protein must be produced to aid in the flow of protons. Finally, the organism must be optimized for resistance to light exposure and to infection.

Many genetic techniques have already been developed for the manipulation of *Synechocystis*. The entire genome has been sequenced and methods are available for inserting new DNA into the genome for introducing specific mutations. It is likely that they will need to co-express the HydGEF proteins (that assist in hydrogenase activation) with the evolved oxygen-tolerant hydrogenase. This will almost certainly need to be done under anaerobic conditions.

Lee and Greenbaum showed that the addition of chemical uncouplers to allow proton leakage across the thylakoid membrane temporarily increased light-dependent hydrogen production by *Chlamydomonas reinhardtii*. The group will controllably express an uncoupler protein that has evolved for that purpose in the brown fat of hibernating animals. Two uncoupler proteins, UCP1 and UCP3, have both been shown to be active for proton leakage when expressed in yeast, and both will be tested

The *Synechocystis* ferredoxin gene will be cloned behind the tryptophan lactose promoter hybrid promoter for controlled expression after induction following isopropyl thiogalactoside addition. They will insert the expression cassette into the *Synechocystis* genome by homologous recombination. The required level of overexpression will be judged by the difference observed in hydrogen production with different levels of induction of ferredoxin overexpression.

Once the hydrogenase, uncoupler protein, and ferredoxin have been expressed in *Synechocystis*, the hydrogen production capabilities of the organism will be tested under photobioreactor type conditions. They intend to measure both the light capturing ability of the organism as well as the hydrogen evolved from the engineered system.

References

1. Flynn, T., *et al.*, "Accumulation of O₂-tolerant phenotypes in H₂-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection," *Intn'l J of Hydrogen Energy*, 27:1421-30, 2002.
2. Koksharova, O. & Wolk, C., "Genetic tools for Cyanobacteria," *Appl. Microbiol. Biotechn.* 58:123-37, 2002.
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Contact: Swartz@chemeng.stanford.edu