Biohydrogen Generation

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Part I: Engineering a Photosynthetic Bacterium For Efficient Hydrogen Production Through Evolution of an Oxygen Tolerant Hydrogenase

Introduction: The long term goal for this project is to develop efficient and economical technology for the biological conversion of solar energy into molecular hydrogen. The first portion of the project seeks to develop an organism/bioreactor system employing a genetically engineered organism that is effective in the direct conversion of sunlight to hydrogen. The organism will use a shuttle protein, ferredoxin, to transfer electrons from the reaction of water photolysis to the hydrogenase enzyme.

The following diagram (Figure 1) shows that this pathway is simple and short and therefore promises to deliver an attractive conversion efficiency. The photosystem of a bacterium such as *Synechocystis* captures sunlight and splits water to generate molecular oxygen, protons, and mobilized electrons. These electrons are transferred to an electron carrying protein, ferredoxin. We propose to introduce into the cyanobacterium a new hydrogenase enzyme that will accept the electrons from ferredoxin and combine them with the protons to make molecular hydrogen. However, the first and major problem is that hydrogenase enzymes are inactivated by molecular oxygen. Thus, the initial focus of this part of the project is to establish protein evolution methods capable of evolving a highly active hydrogenase (such as the one from *Clostridium pasteurianum* (1)) to be insensitive to inactivation by molecular oxygen.

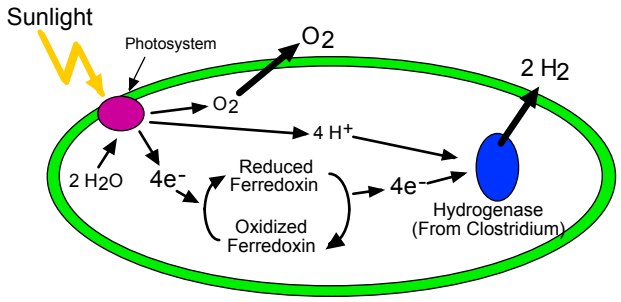

Figure 1: Proposed Engineered *Synechocystis* Bacterium

Approach: Based on the 3-D structure (1) and the molecular properties of the Fe-S hydrogenase (CpI), from *Clostridium pasteurianum* we propose to modify this enzyme to become oxygen tolerant. We believe that it is possible to engineer an altered enzyme structure so that molecular oxygen is sterically excluded from the Fe-S cluster at the active site but that hydrogen can still diffuse away. The predictive capability for protein folding does not allow us to *a priori* select the amino acid changes that would provide such a change. Instead it is now a well established strategy to pursue a protocol called "protein evolution".

For protein evolution to be successful, we must generate genetic diversity around the initial DNA sequence that encodes for expression of the protein. This part is generally reasonably straightforward. The more difficult challenge is establishing methods for searching through 10's of thousands of candidate proteins to find the few that have the new property of oxygen tolerance. If we can develop this capability, we can then iteratively search for enzymes with better and better oxygen tolerance. Fortunately, we believe that our laboratories have a unique set of skills that when combined will allow us to conduct this search relatively quickly and effectively.

The major enabling capability is cell-free protein synthesis. Using this approach, we potentially can synthesize a number of hydrogenase candidates in each well of 96-well microtiter plates. We can also establish procedures that will let us process many 10's of plates per day. Each candidate protein will have an extension that will absorb onto the wall of the microtiter plate well. When the reaction is completed, the well will be washed clean of the reaction solution, but the product hydrogenase will be retained. Then, we will monitor the extent of reaction using reduced ferredoxin as the source of electrons, and conduct the reaction under controlled partial pressures of oxygen.

$$2 \text{ Reduced Ferredoxin} \rightarrow 2 \text{ Oxidized Ferredoxin} + H_2$$

To ensure that we are conducting the authentic reaction desired for the photosynthetic *Synechocystis* strain, we will use the *Synechocystis* ferredoxin (in the reduced form) to supply the electrons required for hydrogen generation. Thus, to implement our search, we must establish the following capabilities:

1. Production, purification, and reduction of *Synechocystis* ferredoxin,

2. Ability to generate diversity in the hydrogenase gene and to conveniently provide DNA templates for cell-free synthesis in 96-well plates,

3. Ability to express active hydrogenase enzyme in the cell-free system,

4. Ability to purify (retain) the expressed hydrogenase in the microtiter plate well,

5. Ability to sensitively detect activity and increased oxygen tolerance, and

2. Ability to recover the DNA that encodes for the improved hydrogenase.

Although this is a significant list of tasks, we have made excellent progress as described on the following page.

Results:

1) Ferredoxin production: The gene that encodes the *Synechocystis* ferredoxin protein has been cloned into plasmids for both *in vivo* and cell-free expression. A 4-liter fermentation of the *in vivo* expression organism was followed by a newly developed purification procedure to yield approximately 8 mg of ferredoxin that appears pure by the conventional indication of a Coomasie-blue stained electrophoresis gel. The purified ferredoxin displays the characteristic light adsorption spectrum (2) and can be reversibly reduced and reoxidized. It also displays the characteristic reddish-brown color. Every indication suggests that it will be adequate to activate the hydrogenase in our protein evolution program.

Since the iron-sulfur center in ferredoxin is similar to four of the centers in our target hydrogenase, we decided to use the ferredoxin as a model protein to develop better methods for the cell-free expression and *in vitro* assembly of active Fe-S clusters in proteins. We used a cell extract derived from an *E. coli* derivative carrying a plasmid that encodes for a number of proteins believed to assist in the assembly and installation of Fe-S centers (3). Under both

aerobic and anaerobic conditions, this extract catalyzed the expression of approximately 200 µg/ml of ferredoxin protein that displays the characteristic color and adsorption spectrum of fully active ferredoxin. At this point, this is a preliminary result and needs further investigation, but the production of apparently active ferredoxin in our cell-free system is a very encouraging indication.

2) Providing DNA Templates for Hydrogenase Production in 96-well Plates: The goal is to be able to produce genetic diversity in the hydrogenase gene, to add a few copies to each well, to use PCR primer extension methods to add the promoter and terminator regions needed for protein expression, and to then amplify the DNA concentration to the level needed for effective protein expression. Significant work on other projects in the Swartz lab has developed and demonstrated each of these steps. By using a genetically modified cell extract, we have also achieved efficient protein expression while using such PCR products as expression templates. We will now develop methods that ensure that the desired number of DNA molecules can be placed in each well and that the mix of protein products faithfully reflects the mix of DNA templates.

3) Expressing Active Hydrogenase in a Cell-Free System: This is by far the most significant challenge for the project at this point and therefore has received the most attention. The gene encoding the CpI hydrogenase was cloned into expression plasmids for both *in vivo* and cell-free expression. Initial evaluation indicated modest expression of hydrogenase polypeptide in the cell-free system, but no activity was formed. It was found that the protein could be expressed with a his_6 purification affinity tag either at the N-terminus or the C-terminus of the protein. However, the yields were lower than expected.

Examination of the specific codons used for each amino acid in the protein showed that many were codons not frequently used by *E. coli*. This was judged to be a potential problem since the transfer RNA mixture we use is derived from *E.coli* and would not be expected to have enough of the specific tRNA's required to recognize these codons. Attempts to augment the tRNA mix were somewhat helpful, but were cumbersome. We therefore designed a completely new gene containing the preferred codons for *E.coli* and synthesized this gene using primer overlap PCR extension reactions. The use of the new gene approximately doubled protein yields but still produced no active hydrogenase. This continued to be the case even when carbamoyl phosphate, ferric ammonium sulfate, and sodium sulfide were added as sources of the chemical entities required to assemble and localize the iron sulfur centers.

To assist in hydrogenase folding, two measures are being developed. The first uses the *E.coli* cell extract from the culture that over-expresses the proteins important for forming the Fe-S cluster in ferredoxin. The second adds a cell extract from the Clostridial strain that naturally makes the hydrogenase in the hope that important helper proteins will be present in that extract. The problem with the second approach is that the Clostridial cell extract also carries a strong background of hydrogenase activity. This makes it difficult to determine if new activity

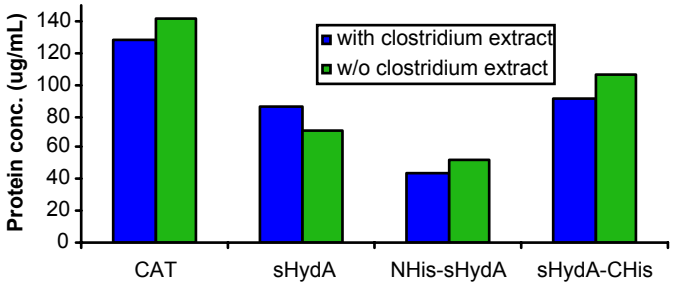

Figure 2: *In vitro* expression of hydrogenase. Cat is chloramphenicol acetyl transferase, the model protein used as an expression comparison; sHydA is the synthetic hydrogenase gene; Nhis-sHydA is the same gene with an N-terminal his_6 extension, and sHydA-Chis is the synthetic hydrogenase gene with a C-terminal his_6 extension

has been formed in the cell-free reaction. Nonetheless, the following figure shows that significant hydrogenase polypeptide is expressed in these systems. In all cases, the modified *E.coli* cell extract was used and the Clostridial extract was added where indicated (Fig. 2).

Significant protein synthesis was obtained in all cases, but so far we have no firm evidence that new hydrogenase activity was formed. There are early indications that a metal chelation adsorbent capable of binding the his_6 tag does retain new hydrogenase activity that was produced in the reactions without the Clostridium extract. This is promising but very preliminary data and needs more work for confirmation.

To provide greater assurance of developing a durable cell-free system for hydrogenase expression and activation, we are now pursuing two additional measures. For the first we will investigate using Clostridial extracts to catalyze the cell-free synthesis of the hydrogenase. Secondly, we are pursuing methods to develop an affinity purification reagent that will allow us to remove the major hydrogenase enzyme from the Clostridial extract. This same reagent should allow us to purify native hydrogenase to use as a comparison standard.

4) Retention of His-tagged Product in the Microtiter Plate Wells: Using a model protein with a his tag, we have demonstrated that this adsorption technique works well even in an anaerobic environment. (There was some concern that the chelation metal used for his tag adsorption would alter its valency state under anaerobic conditions.)

5) Ability to Sensitively Detect Hydrogenase Activity: This stage of the project has not yet begun.

6) Ability to Recover the DNA Encoding Improved Hydrogenases: This work has not begun.

References

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Part II: Genetic Engineering of Photosynthetic and Nitrogen-fixing Cyanobacteria for the Production of Molecular Hydrogen

Introduction:

Since the engineering of an oxygen tolerant hydrogenase as described in Part1 is a significant experimental challenge, we are exploring an alternative approach, in which we direct photosynthetic hydrogen production to an anoxic compartment (heterocysts) found in filamentous cyanobacteria (Figure 3). In filamentous cyanobacteria, photolytic water cleavage is separated spatially from oxygen-sensitive nitrogen fixation. Light-dependent oxygen release proceeds only

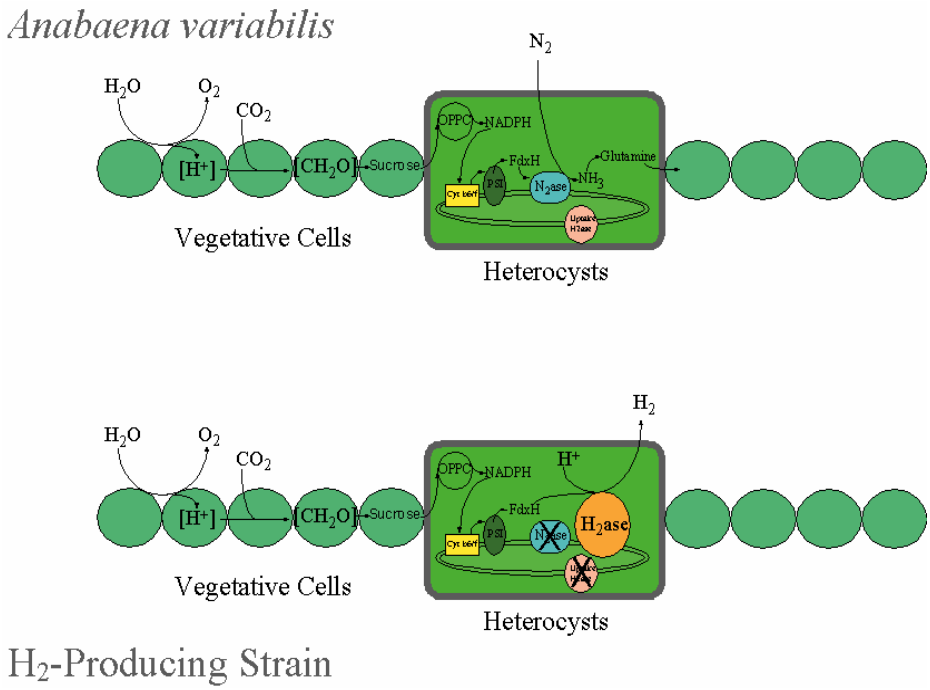

Figure 3. Schematic illustration of photosynthesis and nitrogen fixation in filamentous cyanobacteria such as *Anabaena variabilis* (top panel). Proposed scheme for the conversion of *Anabaena variabilis* to a hydrogen-producing strain (lower panel).

in vegetative cells, whereas nitrogen fixation is restricted to the anoxic heterocysts (Fig. 3, upper panel). Within heterocysts, oxygenic photosynthesis is suppressed. This, together with other oxygen-scavenging mechanisms provides an anoxic environment for the proper functioning of nitrogenase. The remaining vegetative cells (Fig. 3, upper panel) can perform oxygenic photosynthesis, storing light energy into fixed carbon such as sucrose. The sucrose then can be transported into the heterocysts to fuel nitrogen fixation.

Main goals of this approach are:

1) Modify the metabolism of existing photosynthetic and nitrogen-fixing cyanobacteria to produce molecular hydrogen using light energy;

2) Determine the efficiency of the light-driven hydrogen production;

3) Identify rate-limiting step(s) in the light-driven hydrogen production and devise "fixes" to improve the light conversion efficiency.

Approach:

Hydrogenase will be expressed in the heterocysts. A minimal set of three changes is needed to transform a filamentous cyanobacterium into a hydrogen-producing reactor. The specific changes are:

1) Introduce a functional hydrogenase gene into *Anabena* sp.;

2) Express hydrogenase in heterocyst;

3) Delete uptake hydrogenase genes (to prevent the oxidation of newly produced molecular hydrogen).

Results:

1) A new expression vector (circular plasmid DNA) has been constructed. This vector utilizes a nitrogenase promoter, P*nifH*, to drive the expression of the foreign hydrogenase gene. Transcriptional activity of P*nifH* is tightly regulated and is coupled to the developmental program of filamentous cyanobacteria. The present expression vector, therefore, will allow us to target the expression of hydrogenase exclusively to mature heterocysts. In addition, the higher copy number of the vector and the high transcription rate P*nifH* will allow us to express hydrogenase at high level in heterocysts.

2) A DNA construct for the deletion of uptake hydrogenase genes in *A. variabilis* has been made. We will use this construct to delete the uptake hydrogenase gene. This will block the recapture of molecular hydrogen by uptake hydrogenase (which oxidizes molecular hydrogen to protons) in cells that are actively synthesizing molecular hydrogen.

3) A DNA vector carrying three DNA methyltransferases from *A. variabilis* has been constructed. This DNA vector will provide an important function to allow us to introduce DNA into *A. variabilis* at high efficiency. In *A. variabilis*, as in many bacteria, restriction enzymes are present to degrade foreign DNA as part of the natural defense mechanism against foreign or viral DNA. To enable our DNA constructs to enter the cyanobacterium at higher efficiency, we will use the DNA vector to pre-methylate DNA so that it evades the DNA restriction system when it is introduced into *A. variabilis* .

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