II.1.1 BioHydrogen Generation by Genetically Engineered Microorganisms

Part I: Engineering a Direct Pathway for Hydrogen Generation

Investigators
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Introduction

The longterm 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 (Part I) 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 offers attractive conversion efficiencies. 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 existing hydrogenase enzymes are inactivated by molecular oxygen. Thus, the initial focus is to evolve a highly active hydrogenase to be resistant to inactivation by molecular oxygen.

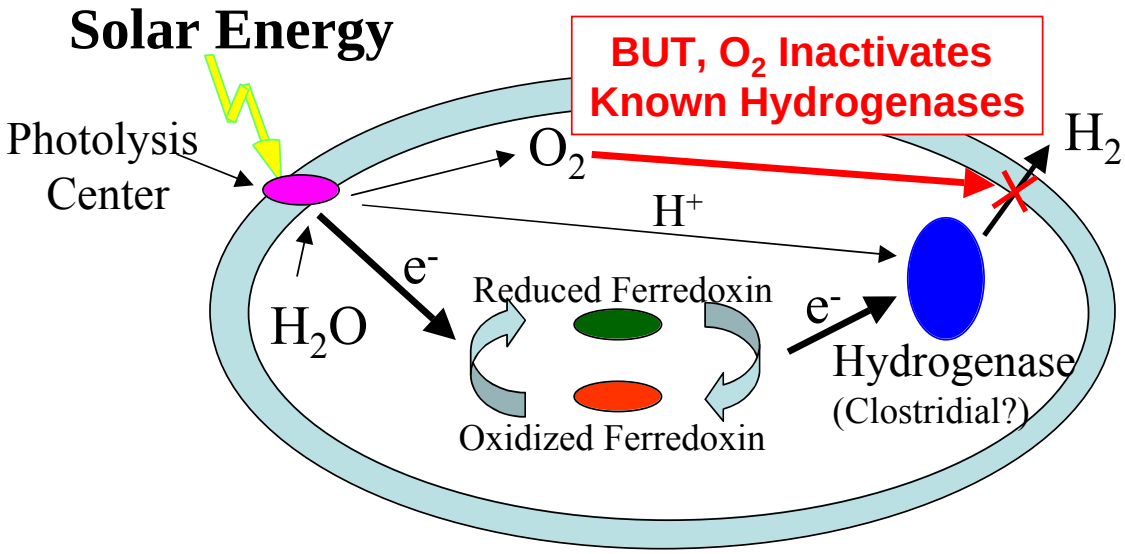

Figure 1: Diagram of proposed *Synechocystis* bacterium engineered to support a short, efficient pathway for the conversion of sunlight and water into molecular hydrogen. As shown, the dominant barrier to this technology is the availability of an oxygen-tolerant hydrogenase enzyme.

Background

A recent Sandia Labs study suggests that the sunlight incident on a 100 square mile area in southern Nevada could supply all of the U.S. energy needs. This is equivalent to only 1.7% of the current U.S. cropland area! However, we need an efficient and cost effective method to convert that sunlight into useable energy. Ideally, a significant fraction would be converted into a clean, portable fuel such as hydrogen.

Therefore, we are committed to the development of an efficient biological process to produce hydrogen from sunlight and water. The power of modern genetic engineering techniques, the proven success of protein evolution technologies, and the extensive knowledge of photosynthesis and bacterial metabolism combine to suggest the feasibility of our proposed project. The proposed reaction system is envisioned approximately as shown in Figure 2.

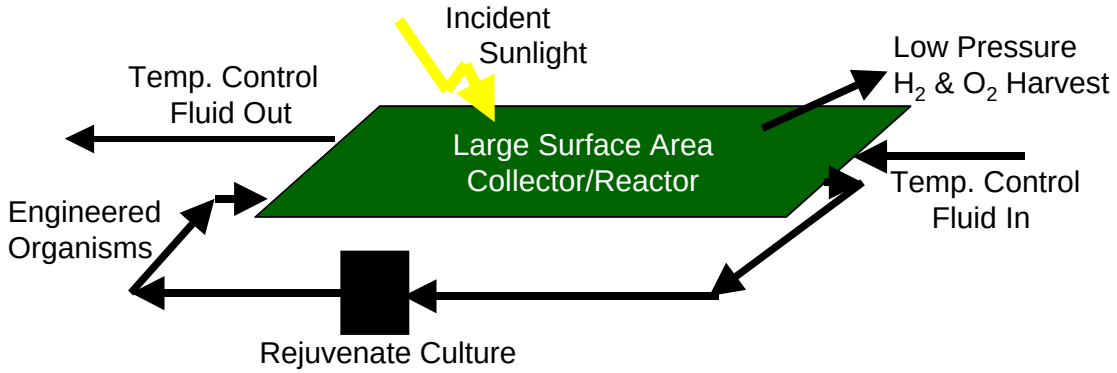

Figure 2: Diagram of a proposed production system for hydrogen production.

The heart of the system is a very large surface area collector/reactor designed to collect the sunlight, remove the evolved gases (H_2 and O_2), control system temperature, and provide optimal mixing patterns. Initial calculations suggest a relatively thin bioreaction layer (less than 1 cm.) is required for the culture to completely absorb available energy. This culture would continually flow through the reactor using a static mixing design to quickly cycle individual organisms between bright and dark conditions (at the top and bottom of the culture channel). The cyclic light exposure will be designed to avoid damage to the light adsorption complexes in the microorganisms caused by constant exposure to bright sunlight. The top of the bioreaction channel would be bounded by a transparent gas permeable membrane. This membrane would allow the evolved H_2 and O_2 to be removed into an upper channel (maintained under vacuum) to maintain low H_2 and O_2 partial pressures in the bioreaction channel. A bottom channel below the reaction channel will carry the temperature control fluid.

As exciting as this prospect is, it is totally dependent upon the availability of a new hydrogenase enzyme that remains active in the presence of the oxygen. Developing such an enzyme is now our primary objective for Part I. WE ARE NOT AWARE OF ANY OTHER SERIOUS EFFORTS TOWARD THIS OBJECTIVE. Because the 3-D structure of the Fe-S hydrogenase (CpI), from *Clostridium pasteurianum* was known (Ref. 1), because it couples to ferredoxin with high turnover numbers, and because it is an

iron-sulfur hydrogenase with a somewhat simpler active site structure than for Ni-Fe hydrogenases, we chose that enzyme as our first target for evolution to oxygen tolerance.

Current technology does not allow us to *a priori* select the amino acid changes that would provide the oxygen tolerance. Instead, it is now a well-established strategy to pursue a protocol called "protein evolution". We first must generate genetic diversity within the initial DNA sequence that encodes for expression of the protein. This part is generally 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.

For the protein evolution to proceed quickly and effectively, we will capitalize on the many advantages offered by cell-free protein synthesis technology. 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 can assess the activity of the hydrogenase by taking advantage of the nature of the hydrogen producing reaction:

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

If the hydrogenase retains its activity in a controlled oxygen environment, hydrogen production activity will be driven by the reduced ferredoxin, protons will be consumed, and the pH will rise. The pH rise can easily be detected using a colored pH indicator dye. 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 and reliably 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
6. Ability to recover the DNA that encodes for the improved hydrogenase.

As reported last year, we quickly accomplished objective number one. Objective number two proved to be significantly more difficult than most people would expect. It requires reliable DNA replication using the polymerase chain reaction to produce 10^{12} identical DNA molecules from a single molecule of DNA. Furthermore, this amplification must be achieved with identical yields for every mutated gene. This is truly "pushing the envelope" in terms of DNA replication technology. However, Keith

Gneshin has made excellent progress and is nearly complete in providing the technology we need (see Results; Part A).

Objective three, the expression of active hydrogenase has proven to be very difficult. Chia-Wei Wang has identified conditions that provide a hint of activity, but not enough for an evolution program. These efforts are summarized in the section entitled: Results, Part B. The lack of success motivated a project by Marcus Boyer to investigate the cell-free synthesis of the *Synechocystis* ferredoxin, a smaller and simpler Fe-S protein. This effort has been very successful as detailed in Results: Part C. The ferredoxin success suggests the feasibility for producing more complex Fe-S proteins, but also suggests that we should broaden our search to investigate the folding of other hydrogenases that are somewhat simpler in structure than the Clostridial enzyme. Objective four was also investigated by Chia-Wei Wang to suggest that the affinity afforded by the commonly used his_6 purification tag with metal chelation adsorption is probably not sufficient (also discussed in Results: Part B). Systems with higher affinity will now be evaluated. The last two objectives have not been addressed as yet but are not expected to be difficult. Our goal now is to demonstrate successful hydrogenase evolution and initial hydrogen generation by *Synechocystis* by the end of next year.

Results: A) Reliable PCR Amplification of single DNA molecules (Keith Gneshin)

Objectives: The success of the hydrogenase evolution project depends, in part, on our ability to fairly evaluate each candidate mutant. To accomplish this, we have carefully focused on the initial stage of the screen which is intended to utilize the polymerase chain reaction (PCR) to amplify every individual molecule of DNA into upwards of one trillion copies of the original template.

Our objectives in this work have been:

1) to ensure that every mutant DNA template introduced into the screen is fully amplified,
2) to ensure that every mutant DNA template is amplified equally relative to other templates in the screen and
3) to devise a complete protocol that ensures reproducible amplification.

The first objective requires a PCR protocol that minimizes the random loss of library members. While some template degradation is inevitable prior to and during PCR, we want to maximize the probability that we can evaluate each and every mutated DNA sequence. Our second objective is to create a PCR protocol that minimizes amplification bias between various members of our library both within a screening well and between separate wells. A final objective is to develop DNA handling protocols, including such tasks as sample transfer, DNA purification, and DNA storage, which ensure reproducible results when the actual PCR is performed. We believe these objectives, when realized, will maximize our ability to identify the very rare but very important mutations that will improve enzyme performance.

Understanding the importance of these three objectives requires a consideration of the intended goal of the hydrogenase screen. Our stated goal of developing an oxygen-tolerant hydrogenase enzyme will require exploring a large number of mutant proteins to find the most beneficial combination of individual mutations. The expected method for generating this library of hydrogenase mutants is family gene shuffling, a recombinant PCR method that allows multiple related genes to be combined in a manner that mimics *in vivo* gene recombination. The biggest benefit of family gene shuffling is that it pools together the mutations within a single gene that have already been selected as beneficial by the source organisms. In this respect, the variants explored are more likely to contain complementary mutations rather than deleterious random ones. When compared to methods of random gene mutation such as error-prone PCR, the speed at which desired proteins are isolated from a gene library is much faster via gene shuffling.

A gene sequence that leads to an oxygen-tolerant hydrogenase is expected to be very rare. Because of this, we are developing a high-throughput screening method that allows us to very specifically isolate a member of a gene mutation library and test the activity of the resultant enzyme. A basic diagram of our proposed screening method is shown in Figure 3. Following the generation of our hydrogenase library by family shuffling, mutants will be diluted to a very low concentration then dispensed into individual screening wells with

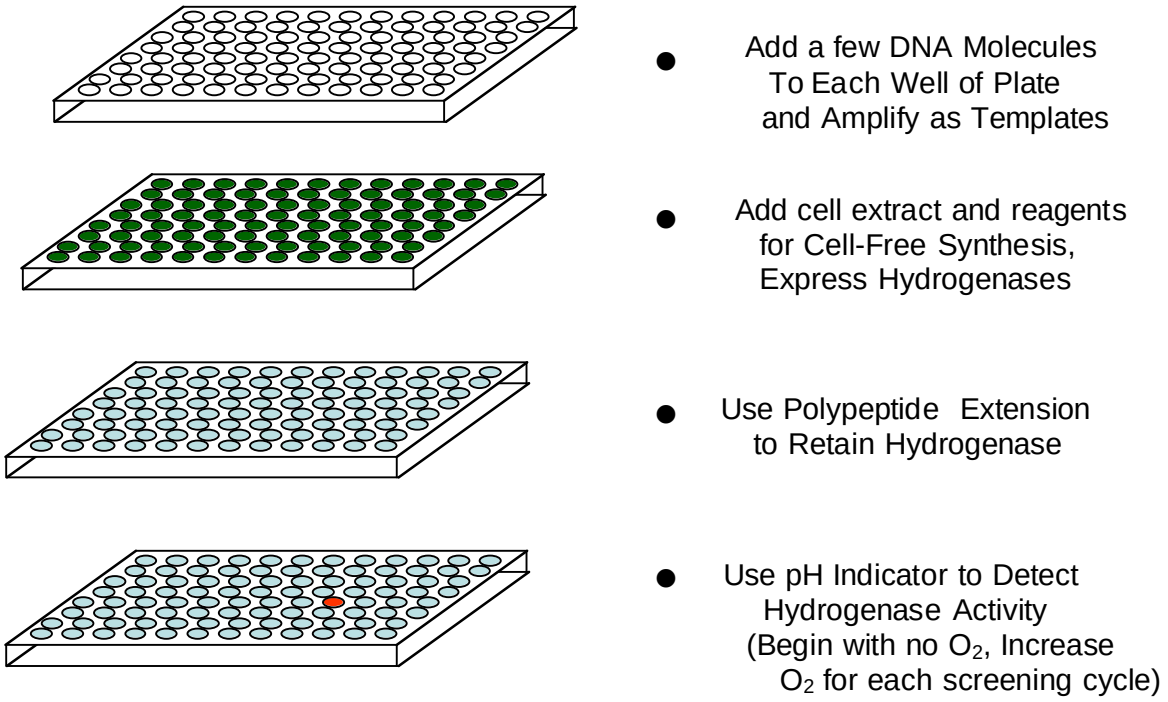

Figure 3: Approach to high-throughput screening using PCR and cell-free protein synthesis in multi-well microtiter plates.

a single DNA encoding each of approximately five to ten mutants per well. Once the library has been divided up, PCR reagents can be added to each well. From the initial DNA templates, each mutant will be amplified by a factor of 10^{12}. These PCR products will serve as templates for coupled transcription and translation by the cell-free protein

synthesis technique employed in our laboratory. Our current work in developing the previously described screening method centers on the polymerase chain reaction. We believe this aspect of the screen warrants the most attention because the ability of cell-free protein synthesis to occur is entirely dependent upon having adequate amounts of DNA template to transcribe and translate into protein.

Cell-free protein synthesis and enzymatic activity assays are methods that have been developed and employed in our laboratory. However PCR driven DNA amplification from very low DNA concentrations is a sensitive task that has required significant and careful study. As detailed below, initial work showed that routine PCR approaches failed miserably in this much more demanding task.

Initial Problems with PCR Protocol Have Been Resolved. The development of a PCR protocol for our high throughput screen has been a difficult problem that has led to some surprising findings. Although this aspect of our work has taken longer than anticipated, we are very close to having a reproducible procedure in place for amplifying single molecules of DNA as required in our screening scheme. While PCR has been a ubiquitous technique in biochemistry for nearly 20 years, our screen demands much more of the PCR reaction than is typically required. Most PCR reactions are performed at high starting template concentrations, requiring only up to one million-fold amplification of the target sequence. The hydrogenase screen will require an additional six orders of magnitude of amplification. This additional amplification more than doubles the length of the reaction. At such long time scales, the stability of PCR reagents is tested and minor perturbations for a typical reaction will cause the failure of the longer, more demanding amplificaiton. These difficulties have posed challenges to our development of the high-throughput screen. For example, we have encountered: a) the thermal degradation of linear DNA templates leading to low yields or reaction failure, and b) a large variety of aberrant reaction products caused by poor primer characteristics, poor purification reagents, and metal contamination. We have already found technical solutions to all of these problems and believe we only need to develop an effective template dilution method to complete this work.

Prior to attempting single-molecule PCR, we wanted to demonstrate an effective protocol for amplifying high concentrations of initial DNA template, with upwards of ten million starting copies. While this is a fairly common initial concentration for PCR, we encountered initial difficulty because our starting templates were linear DNA rather than circular plasmid DNA. A literature search revealed a mechanism for thermal degradation that severely affected linear DNA templates. The solution to this problem was to drastically reduce the duration of the high temperature steps in the PCR protocol. Successive PCR reactions showed highly-reproducible amplification for a wide range of initial template concentrations.

Ensuing work focused largely on locating an effective primer for single-molecule PCR. We initially used a unique primer to amplify each strand of the double-stranded DNA template (in this case, the sHydA gene encoding for the clostridial hydrogenase). These primers worked well for the original gene sequence, but the primers tended to

produce improper, shortened products for mutated versions of the gene, as shown in Figure 4A. Each lane shows the reaction products for the hydrogenase gene with the same restriction site sequence located in a different place in the gene. The lower bands represent undesired side products. The improper products were attributed to mispriming of the template sequence. We solved this problem by converting to a single-primer format. By using only a single primer to initiate replication for both DNA strands and optimizing its sequence, we were able to develop a primer that had minimal complementarity to the gene sequence outside of the intended priming sites, minimizing the chances of mispriming. This technique also had the advantage of allowing us to choose a primer with a favorable high annealing temperature and minimal self-complementarity, reducing the chances of primer-dimer formation which can also lead to aberrant PCR products. Single-primer PCR was far more reliable than the original double-primer PCR for amplifying a series of unique mutants of our hydrogenase gene, as shown in Figure 4B.

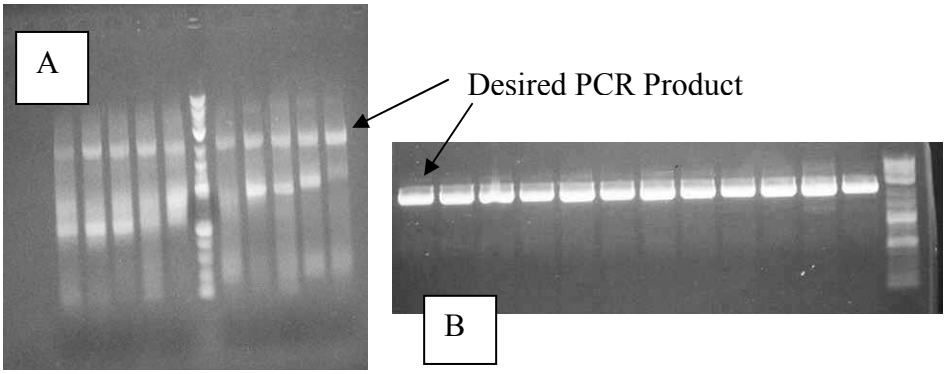

Figure 4: A) Aberrant products developed from hydrogenase mutant genes during double-primer PCR B) Elimination of aberrant products by single-primer PCR.

We also found DNA purification to be problematic during the course of our early PCR work. To demonstrate the reproducibility of a PCR protocol, we needed to transfer the products from an initial reaction into a successive set of PCR reactions. Before the actual DNA transfer to the new reactions, we would attempt to purify the DNA products to remove any deleterious carryover from the previous reactions. Results in the second PCR were initially variable; the source of the problem was ultimately traced to our purification protocol. At the time, spin columns were used to trap DNA while other PCR reactants were washed away. It was found that spin columns tended to completely fail at the rate of 1 in 6 attempts, even when purifications were carried out in parallel with identical reagents, as shown in Figure 5. We never successfully determined what aspect of this purification technique was failing. We avoided the problem by adopting the more labor-intensive but more effective technique of phenol-chloroform extraction of DNA. With this purification method, we were able to carry DNA through a series of reactions without failures. While this purification problem was ancillary to the actual PCR protocol, it nonetheless represents a major factor in ensuring successful amplification of DNA.

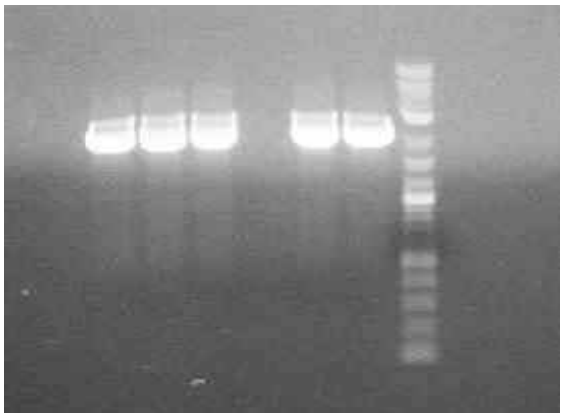

Figure 5: Spin column purification failure. Six PCR reactions were conducted in parallel and purified. The reaction run in lane 4 was successful but the purification failed completely.

A final major problem that we have encountered is metal contamination. This problem has only become apparent now that we have begun to actually attempt amplification from a single molecule of DNA. At very low template concentrations, we saw only two results: successful amplification of our hydrogenase template or an undesired short product. An example of these results is shown in Figure 6. At no point did we see these two products occurring simultaneously within a single reaction,

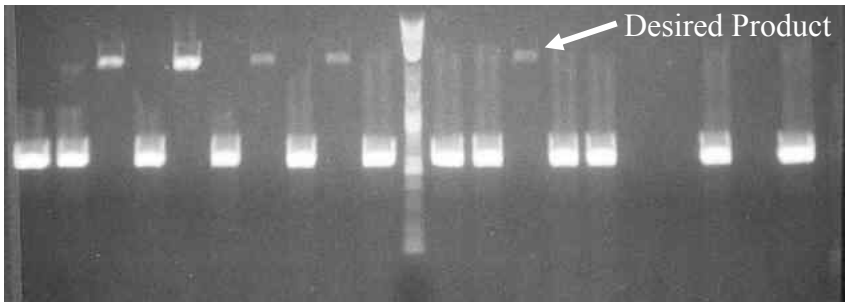

Figure 6: Primer-dimer accumulation in single-molecule PCR. Left lanes are reactions performed from an approximate starting concentration of 10 molecules of template per reaction. Right lanes began with approximately 1 molecule per reaction.

suggesting that they were in competition with each other for available primers during the early stages of the amplification. An initial speculation was that a trace metal contamination could have caused our primers to associate with each other just enough to cause a primer dimer to form. Control reactions without any DNA template showed none of the aberrant product (see Figure 7). We suspected that the potential source of metal contamination was the DNA template itself. To test this hypothesis we added EDTA, a metal-chelating agent, to our DNA before adding it into the PCR reaction. The result was a complete elimination of the aberrant product, shown in Figure 7. Under typical conditions, the EDTA is capable of suppressing primer dimer formation to allow reliable

amplification of our hydrogenase gene. The very light product band in lanes 6-10 suggests that further work is needed for reliable amplification from single DNA molecules, but clearly the primer dimer threat has been dramatically reduced.

Figure 7: Elimination of primer-dimer accumulation with EDTA. Lanes 1-10 contain 1.0 μM EDTA. Lanes 1-5 have 100 templates per reaction initially, and lanes 6-10 have 10 templates. Lanes 11-20 contain no EDTA. Lanes 11-15 have 100 templates initially and lanes 16-20 have 10 templates. Lanes 21-25 are no-template controls with 1.0 μM EDTA.

With these problems solved, we have made significant progress toward satisfying our DNA amplification objectives. To create a robust PCR technique that minimizes random loss due to thermal degradation, we have chosen an unconventional PCR cycling pattern that greatly minimizes the length of time our template is exposed to high temperatures. Also, in an effort to minimize misannealing, we've chosen a primer that anneals at the extension temperature of the polymerase enzyme thereby eliminating the need for the low-temperature annealing step in the typical PCR cycle. Figure 8 shows the changes we have made to the standard PCR protocol. Despite these significant changes to the PCR protocol, we still get efficient and reproducible amplification of our gene target.

We have also shown that single-primer PCR can amplify mutant genes in a library without significant bias toward any particular members. To do this, we created a series of mutants of our sHydA (synthetic Clostridial hydrogenase) gene, each having ta different location for the recognition sequence for a restriction enzyme. Each member of the mutant library was added in equal concentration to a PCR reaction and then amplified simultaneously. Following the PCR, the product was digested by the appropriate restriction enzyme, causing each mutant to be cut at its unique recognition site. The result was a range of DNA fragments, each of which could be attributed to a particular mutant from the library. Qualitatively, all members of the mutant library were seen in the final digestion product, meaning all were amplified to roughly the same extent as seen in Figure 9. While the qualitative result suggests we can perform PCR without bias toward particular members, we intend to perform a more rigorous quantitative experiment to verify this result.

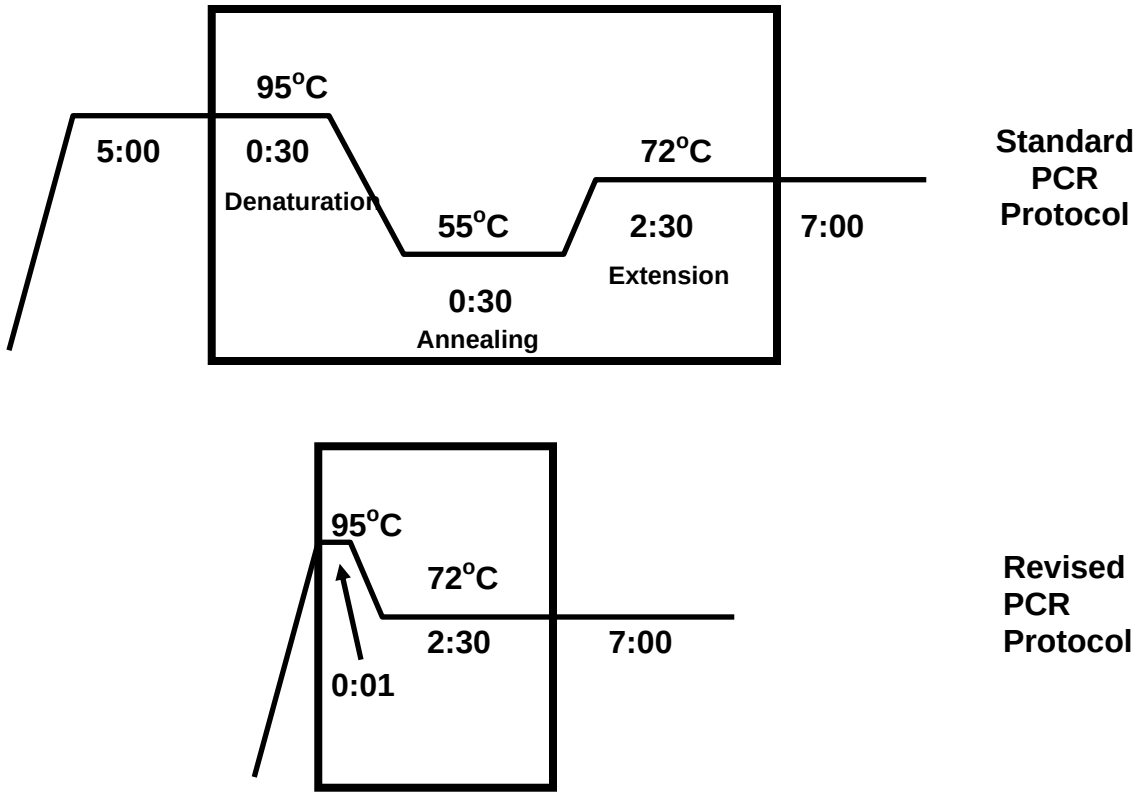

Figure 8: Revision to standard PCR protocol for single-molecule PCR. Times are given in minutes. Boxed regions represent the actual cycling pattern of the PCR, repeated up to 65 times.

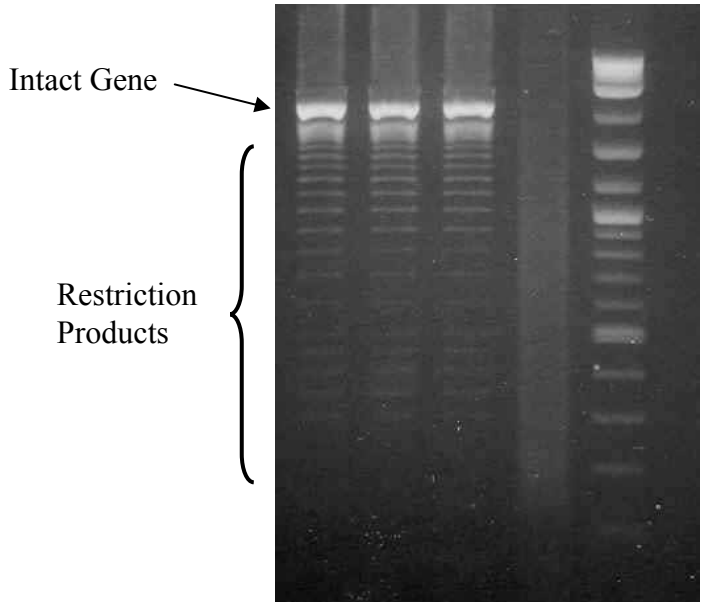

Figure 9: Equality of amplification among coamplified hydrogenase mutants. The brightest band is the actual hydrogenase gene and the shorter bands are fragments from digested genes.

Finally, we are almost to the point of being able to amplify a single-molecule of DNA to a very high concentration. Our recent attempts showed acceptable amplification with approximately 100 molecules of initial template (Figure 7). However, further dilution has produced variable results. We believe that most dilution problems have thus far been tied to metal contamination issues. Now that we know how to mitigate this problem, we believe we will be able to more successfully dilute DNA down to the single molecule level and amplify from there.

Summary and Future Work (Results: Part A). We have developed a robust method for amplifying DNA targets from low concentrations of starting template. In order to do so, we have adopted a unique protocol for our PCR reactions. The abbreviated PCR cycle profile was chosen to increase the success of the PCR procedure, but it has also produced a practical benefit. Our original PCR method would have required nearly six hours to complete when amplifying from the single molecule level. Our current abbreviated protocol requires just over three hours. This time savings now makes it feasible to conduct the entire protein screen, from DNA amplification through activity assay, within a single day. Making each round of screening a one-day process will accelerate our hydrogen evolution project.

Our last remaining issue before we complete this work is perfecting our template dilution method. Once we can reproducibly produce single molecule template concentrations in our PCR reactions, we believe we will have all of the technique in place for the PCR portion of our screen. We will be performing several quantitative experiments soon to verify our results, and we believe this portion of the project will be completed by July 2004. The in-place activity assay is expected to be straightforward. Thus, the remaining obstacle is the cell-free expression of <u>active</u> hydrogenase as discussed in the next section.

Results: B) 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 hydA gene encoding the Clostridial hydrogenase was cloned into expression plasmids for both *in vivo* and cell-free expression. It was found that the protein could be expressed with a his_6 (His) purification affinity tag either at the N-terminus or the C-terminus of the protein. Evaluation to date has indicated moderate expression of the hydrogenase polypeptide in the cell-free system with the extracts from *E. coli* and *Clostridium pasteurianum*, and a tiny amount of activity seems to originate from the newly synthesized HydA with a His tag compared to the control.

As reported last year, initial expression with the native hydrogenase gene suggested a problem with rare codons (codons rarely used by *E.coli*). Therefore, we designed and synthesized a completely new gene sHydA containing the preferred codons for *E. coli* using primer overlap PCR extension reactions. The use of the new gene approximately doubled protein yields but produced no active hydrogenase with only *E. coli* S30 extract 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, we added a cell extract from the Clostridial strain that naturally makes the hydrogenase in the hope that important helper proteins will be provided. The challenge with this approach is that the Clostridial extract also carries a strong background of hydrogenase activity. This makes it difficult to determine if new activity has been formed in the cell-free reaction. To overcome this, we used the clone of HydA with a his_6 tag as our target protein for cell-free synthesis. After the cell-free reactions with the radio-labeled [C^{14}]-leucine, we can quantify the yields of the newly synthesized proteins by measuring the incorporated radioactivity and can remove the background hydrogenase activity by using the his_6 tag to retain the newly synthesized hydrogenase in metal chelation adsorption wells while washing away the other reaction components. We used the clone of HydA with the N-terminal his_6 tag in most experiments since the tag is assumed to be more accessible for purification based on the crystal structure of the enzyme.

In all cases, the standardized *E. coli* S30 cell extract was used and the Clostridial extracts were added in different portions as indicated (Fig. 10). The total protein yields were measured, and the cell-free reaction mixtures were also centrifuged to obtain the soluble fractions for protein quantification. We found that the addition of the Clostridial extracts reduced the yields of the proteins synthesized from the cell-free reactions for both the control protein chloramphenicol acetyl transferase (CAT) and for our target protein, HydA. It is interesting to notice that this inhibitory effect from the addition of the Clostridial extract is even more severe for CAT than for HydA.

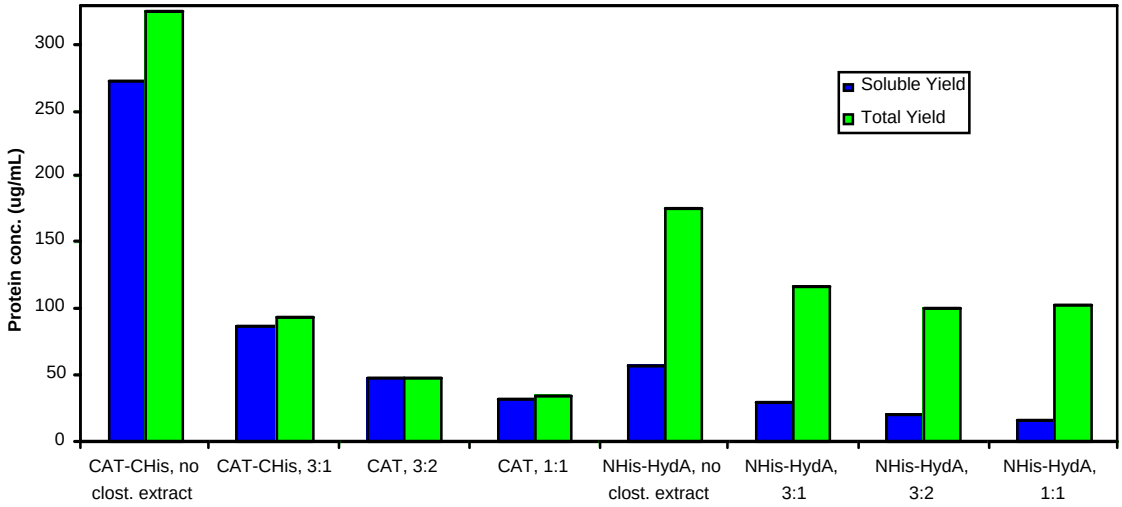

Figure 10: *In vitro* expression of hydrogenase. CAT is used as an expression comparison; CAT-CHis is the CAT protein with a C-terminal his_6 extension. HydA represents the *Clostridium pasteurianum* hydrogenase encoded by the synthetic gene sHydA; NHis-HydA is the same protein with an N-terminal his_6 extension. The volume ratios between *E. coli* and Clostridial extracts in the cell-free system are listed, e.g. 3:1 represents that 3 volumes of *E. coli* extracts were used plus additional 1 volume of Clostridial extract.

Since adequate protein synthesis was obtained in all cases, we proceeded to purify and assay the synthesized his_6-tagged enzymes using commercially available Ni-chelation microtiter plates. The typical procedure includes loading the soluble fractions from the cell-free reaction mixtures into the wells of the plates, incubating to allow the his-tagged proteins to absorb to the walls of the microtiter plate wells, and washing to remove the background proteins. Finally, we can elute out the bound proteins from the wells for protein assays or can add assay reagents directly into the wells to determine enzymatic activity. We added colorless, oxidized methyl viologen into the wells and monitored the formation of the blue color associated with the product of the hydrogenase reaction, reduced methyl viologen. Typical results are presented in Table I. They indicated that the his_6 tag is not effective in retaining low concentrations of soluble product in the wells. However, the results also suggest the formation of hydrogenase activity associated with HydA expression. This is encouraging data, but the activity is much less than would be expected from this quantity of adsorbed hydrogenase if it were fully active. These results are therefore prompting us to examine the expression of alternative, somewhat simpler hydrogenases from blue-green algae.

Table I: Retention in the Ni-chelation microtiter plate wells and hydrogenase activity of the His_6-tagged reaction products produced as in Figure 10. The absorbed protein was eluted from the wells using 250 mM imidazole and the yields were measured by radioactivity. The reduced methyl viologen signal was measured at 560nm (OD560) after overnight incubation to assess hydrogenase activity.

Extract Mix, E. coli : Clostridial	3:0		3:1		3:2		3:3 (1:1)	
Protein of Cell-free Synthesis	CAT	HydA	CAT	HydA	CAT	HydA	CAT	HydA
Eluted Protein (ng)	39.68	20.73	20.77	5.16	0.19	0	2.36	2.22
Methyl Viologen Signal (OD_{560})	0.02	0.10	0.11	0.21	0.12	0.18	0.33	1.07

Retention of His-tagged Product in the Microtiter Plate Wells. The plates we used for purifying the His-tagged proteins were coated with nickel (Ni-NTA) and the dissociation constant Kd of His_6 to Ni-NTA is on the magnitude of 10^{-6} M. In contrast, typical antibody binding affinities have Kd values ranging from 10^{-7} M to 10^{-10} M or lower. Thus, we will evaluate the use of anti-his tag antibodies for product adsorption. For example, the Tetra-His antibody has an dissociation constant, Kd, of 10^{-8} to $5*10^{-8}$, and the Penta-His antibody has a Kd of $5*10^{-8}$ to 10^{-9}. We are now evaluating the Penta-His antibody.

Developing Antibodies for Removal and Purification of the Native Hydrogenase. In addition to the efforts for the expression of active hydrogenase in a cell-free system, we pursued the development of 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. We used the technique of phage display to identify monoclonal antibodies suitable as the affinity purification reagents. First, the sHydA gene with the N-terminal

His-tag was expressed *in vivo* in *E. coli* to prepare large quantities of NHis-HydA proteins. Although not active, the soluble HydA polypeptide can serve as the antigen for antibody screening procedures. The soluble fractions of the expressed HydA protein with the His-tag were purified at a larger scale using the Ni-chelation columns of the His-Trap Kit (Amersham Biosciences, now part of GE Healthcare, Piscataway, NJ).

After dialysis with the PBS buffer, the purified NHis-tagged HydA in PBS was used as the antigen in coating immunotubes. We grew the Tomlinson libraries I & J (MRC Centre for Protein Engineering, UK) and selected bacteriophage against the antigen through three rounds of selection. Phage were screened for binding by polyclonal and monoclonal ELISA to isolate antibody candidates. Finally, we produced soluble monoclonal ScFv antibody fragments in *E. coli* strain HB2151 and purified them using Ni-NTA spin columns. From these phage display procedures, we identified five distinct soluble monoclonal antibodies with diverse CDR (complementarity determining region) sequences of the VH (variable heavy) chain of the antibody. All five can potentially serve as affinity purification reagents.

Summary and Future Work (Results: Part B). We now achieve reliable expression of his_6 tagged Clostridial hydrogenase polypeptides. Conventional metal chelation microtiter plates do not appear to provide sufficiently high adsorption affinity to completely capture the expressed protein. Antibody adsorption will now be tested. Although small amounts of hydrogenase activity have been produced after mixing Clostridial cell extracts into the reaction mixture, the activity levels reflect a very small fraction of the expressed protein. Additional changes in the expression and folding environment will be tested, but it may be that this hydrogenase is too difficult to assemble for present cell-free protocols to be successful. As described below, additional hydrogenases will now be evaluated in parallel to this effort.

Results: C) Cell-free Production of Active Ferredoxin

Executive Summary: Cell-free Production of Ferredoxin

- Hydrogenase is a complex protein that incorporates clusters of inorganic iron and sulfur that make protein maturation a challenge in a cell-free environment
- To better understand conditions favorable to iron-sulfur cluster formation, we have expressed a simpler model iron-sulfur protein called ferredoxin from the cyanobacterium Synechocystis sp PCC 6803.
- Upon supplementation of iron and sulfur sources, the cell-free system can efficiently express and mature ferredoxin to a fully active form
- Increasing the concentration of iron-sulfur cluster helper proteins changed the optimal supplementation levels for the iron and sulfur source molecules, but did not increase the overall yield of active ferredoxin
- When compared with ferredoxin produced in *E.coli*, cell-free ferredoxin is equally active.
- The understanding gained from expression of ferredoxin is being applied toward expression of the more complicated hydrogenase enzyme.

Ferredoxin as a model iron-sulfur protein. Hydrogenase belongs to the family of iron-sulfur (Fe-S) proteins distinguished by their incorporation of inorganic iron-sulfur clusters. These clusters compose the active sites of the proteins and are necessary for their electron transfer activity. Installation of iron-sulfur clusters requires reduced iron and sulfur sources as well as other helper proteins. The expression and maturation of an iron-sulfur protein has not previously been accomplished in any reported cell-free system.

Anticipating the challenge of maturing the complicated Fe-S protein hydrogenase in the cell-free system, we began in parallel the expression and maturation of a simpler Fe-S protein, ferredoxin from the cyanobacterium Synechocystis sp PCC 6803, the organism intended for use in biohydrogen production. By successfully expressing and maturing ferredoxin, we have learned about conditions favorable for Fe-S cluster formation in the cell-free system.

Ferredoxin is a small protein, is highly acidic, and contains one 2Fe-2S cluster. Its homologues serve as electron carriers in many organisms and as electron donors to Fe-type hydrogenases. Synechocystis ferredoxin was found to be highly soluble, and easily purified. An activity assay was developed to measure the activity of ferredoxins synthesized in our lab. For these reasons, ferredoxin has served as an ideal model Fe-S protein for the cell-free system.

No quantitative assay for ferredoxin activity was found in the literature. Therefore, an assay was developed using the following reactions:

$$NADPH + Fd_{ox} \rightarrow Fd_{red} + NADP^{+}$$

$$Fd_{red} + CytC_{ox} \rightarrow Fd_{ox} + CytC_{red} \text{ (colored)}$$

Enzyme and substrate concentrations were adjusted until the rate of CytC reduction was dependent upon the concentration of redox active ferredoxin. This proved to be a useful and highly repeatable method for determining the concentration of competent ferredoxin.

Iron and Cysteine Supplementation. To supply the iron and sulfur for formation of the Fe-S cluster of ferredoxin, we supplement the cell-free reaction with ferrous ammonium sulfate and with the amino acid cysteine. Some Fe-S proteins have been re-folded in vitro using an inorganic sulfur source (Ref 2), but natural systems provide sulfur by the enzymatic desulfuration of cysteine (Ref 3). Figure 11 shows the effect of supplementation of iron and cysteine on the cell-free production of ferredoxin. Addition of iron to a reaction containing the standard level of cysteine provided for protein synthesis had only a small effect on protein production and activity. Addition of cysteine beyond the standard amount without any addition of iron is beneficial for protein production, but has no effect on activity. Supplementation of both iron and cysteine causes a significant increase in both protein production and activity indicating that the effects of these factors are coupled.

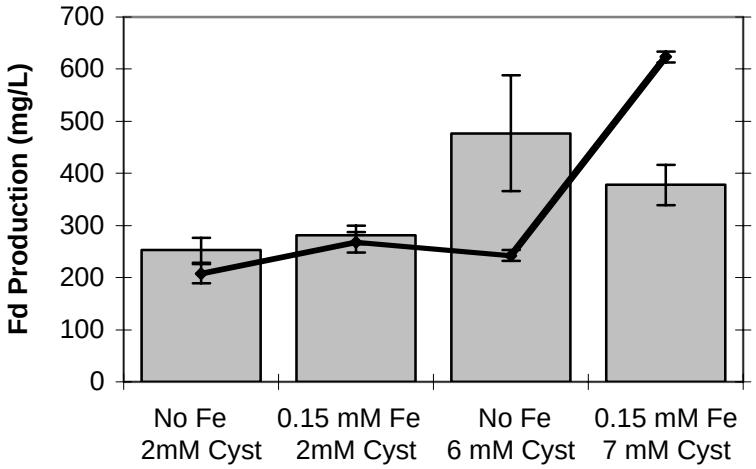

Figure 11: Supplementation of Fe and Cysteine has a synergistic effect on ferredoxin protein production and activity

ISC Helper Proteins. In addition to the presence of iron and sulfur sources, helper proteins are required to assemble and install some iron-sulfur clusters. Nakamura, et. al. have shown that the coexpression of the 9-protein *E. coli*. ISC operon improves yields of ferredoxins produced *in vivo* (Ref 4.) To test whether increasing the concentrations of these proteins would have an effect on the cell-free system, we obtained the plasmid containing the *E. coli* ISC operon. This plasmid was transformed into a cell extract source strain of *E. coli*. Expression was induced during the growth of the extract source cells to increase the abundance of these proteins in the cell-free extract. This extract is referred to as pRKISC extract after the name of the plasmid containing the ISC proteins.

Use of this extract slightly decreased the optimal levels of Fe and cysteine supplementation (Figure 12). However, the total yield and specific activity of ferredoxin produced by this extract were not improved. It would appear that an overrepresentation of ISC helper proteins results in the more efficient use of iron and cysteine, but the same expression and maturation results can be obtained from standard extract by increasing supplemented iron and cysteine concentrations.

Characterization of Purified Ferredoxin. Ferredoxin was produced *in vivo* by overexpression in *E. coli*. This ferredoxin was purified and used as a reference against which to compare the ferredoxin produced in the cell-free system. Ferredoxin was also purified after cell-free synthesis using either the lab standard extract (KC1 Fd) or the extract with overexpression of ISC proteins (pRKISC Fd). The activity, iron content, and protein concentration of purified samples was measured. The activity of both cell-free ferredoxin samples was found to be at least equal to the activity of ferredoxin produced in vivo on a per-iron basis (Figure 13). The amount of iron incorporated per protein in each sample was also very similar. In short, cell-free expression of ferredoxin produces fully active ferredoxin with approximately the same maturation efficiency as recombinant *in vivo* expression in *E. coli*.

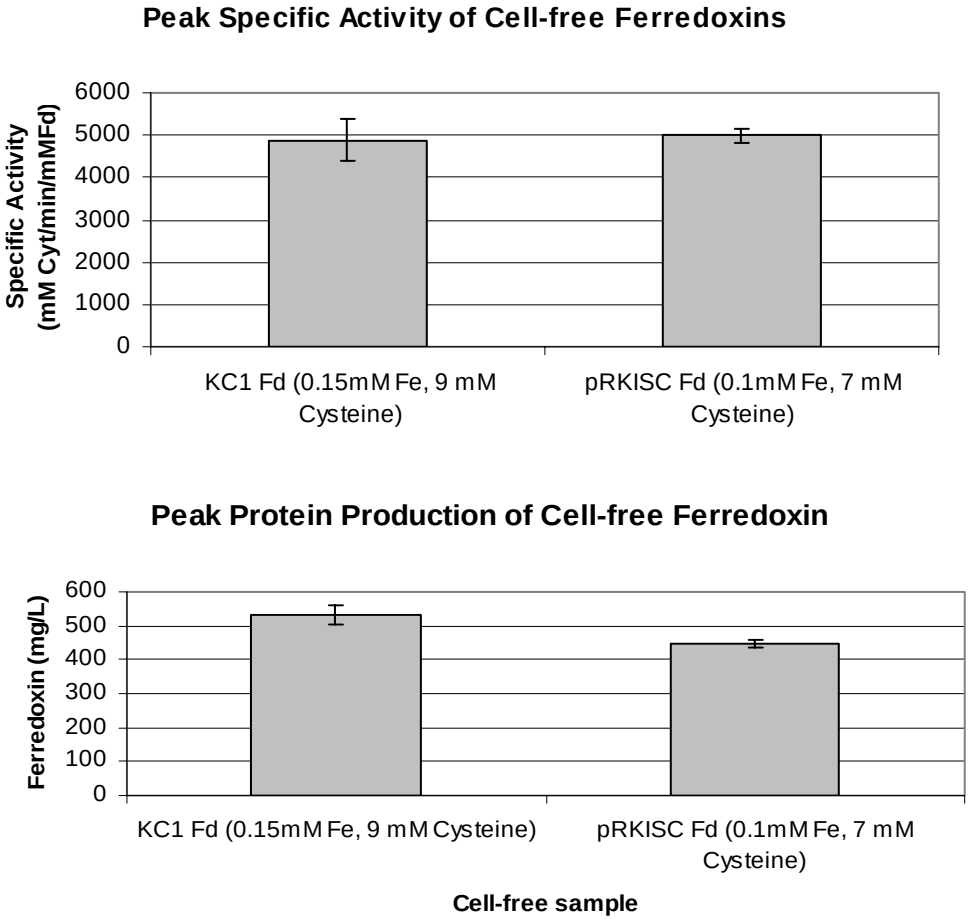

Figure 12: Specific activity and protein production of ferredoxin produced in the cell free system under conditions optimized for lab standard extract (KC1) or extract enriched for ISC helper proteins (pRKISC).

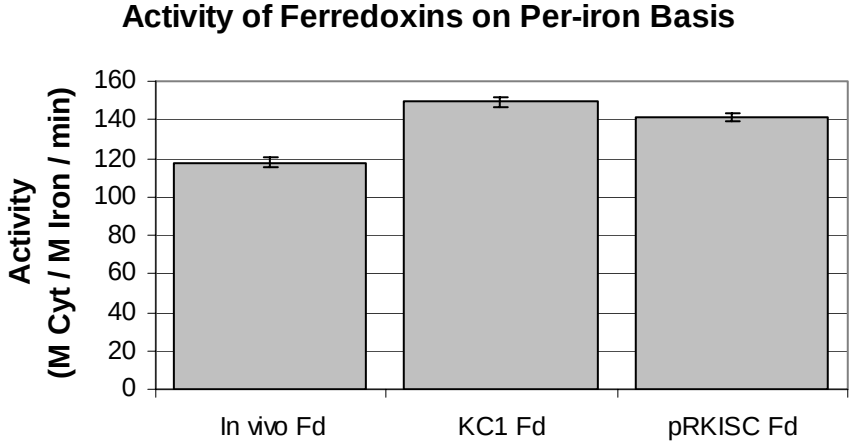

Figure 13: Purified cell-free ferredoxin is as active as ferredoxin produced in *E. coli*.

UV-Vis Absorbance Spectrum of Purified Cell-free Ferredoxin. The UV-Vis absorbance spectrum of cell-free ferredoxin is nearly identical to the published spectra for ferredoxin purified from the native organism Synechocystis sp PCC 6801 (Ref 3). The spectrum shifts upon addition of the reducing agent sodium dithionite, indicating the reduction of the iron-sulfur cluster (See Figure 14). The original spectra shape is recovered after re-oxidation of the iron-sulfur cluster by bubbling with air. Thus, reduction and re-oxidation can be repeated as expected for fully competent ferredoxin.

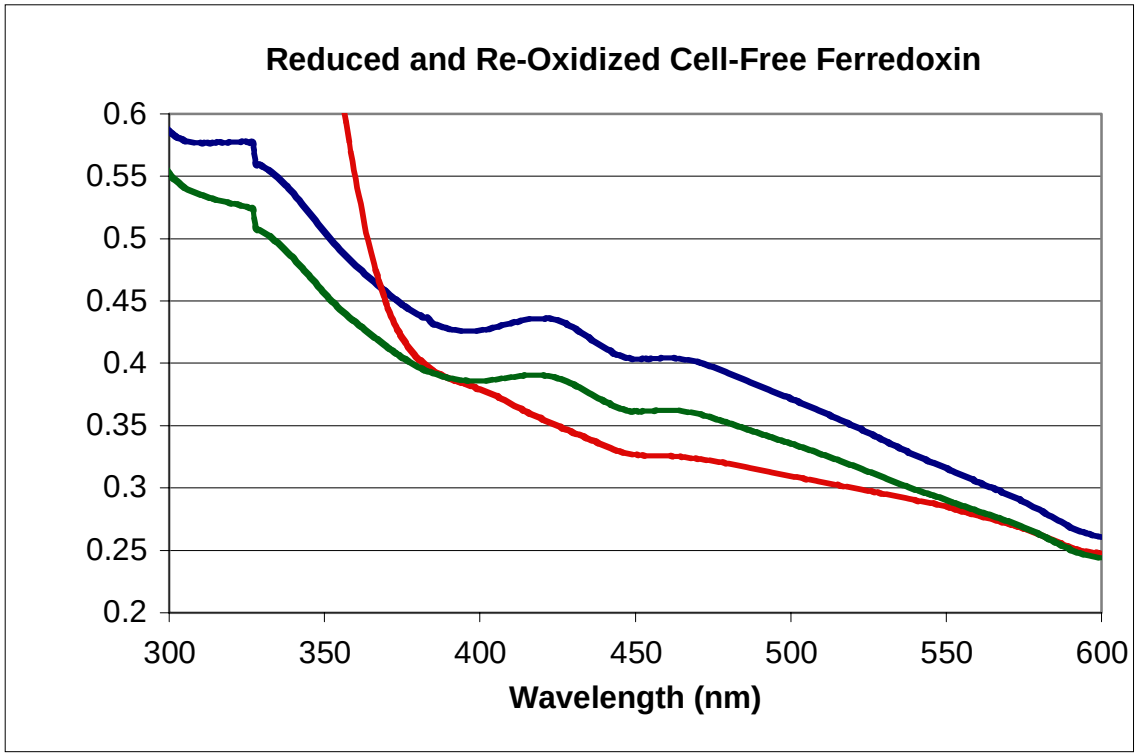

Figure 14: UV-Vis absorbance spectrum of cell-free ferredoxin in oxidized, reduced and re-oxidized condition

Ferredoxin Conclusions. When supplemented with reduced iron and sulfur sources, our *E. coli*-based cell-free system contains all the factors necessary to form and install a simple iron-sulfur cluster into the protein ferredoxin. Maturation of ferredoxin is accomplished with approximately the same efficiency in the cell-free system at 37 °C as was achieved in *E. coli* at 28 °C. Matured cell-free ferredoxin was fully active and capable of passing electrons between known coupling agents. The information gained about the capabilities of the cell-free system and conditions favorable for iron-sulfur cluster formation is to be used toward folding of the more complex iron-sulfur protein hydrogenase.

Progress

Over the past year, we have made significant progress in developing molecular biology methods required for the efficient evolution of an oxygen-tolerant hydrogenase. We have also established, for the first time, that active proteins containing iron-sulfur centers can be produced in cell-free synthesis systems. However, we have yet to demonstrate success in producing an active hydrogenase. This will be the dominant

focus for next year's research. In parallel, we have evaluated information in the literature relative to various feasibility issues for the overall direct conversion approach. We continue to be convinced that if we can develop an oxygen tolerant hydrogenase, hydrogen production economics are favorable for the biological conversion of sunlight to hydrogen.

Future Plans

1. Complete methods development for efficient screening of hydrogenase gene libraries using cell-free protein synthesis.
2. Clone, express, and optimize activation of hydrogenases from blue-green algae.
3. Conduct genetic evolution of oxygen-tolerant hydrogenases.
4. Clone and express native hydrogenase into *Synechocystis* and test for hydrogen production.
5. Clone and express evolved hydrogenase into *Synechocystis* and begin to optimize hydrogen production rates and efficiencies.

Publications

No publications have been submitted to date. The cell-free ferredoxin expression work is now being prepared for publication. The molecular biology improvements are nearly ready for publication.

References

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2) Meyer, J., Moulis, J., and Lutz, M. High-yield chemical assembly of [2Fe2X] (X=S, Se) clusters into spinach apoferredoxin: product characterization by resonance Raman spectroscopy. *Biochimica et Biophysica Acta* 871, 243-249, 1986.
3) Bottin, H. and Lagoutte, B., Ferredoxin and flavodoxin from the cyanobacterium Synechocystis sp PCC 6803. *Biochimica et Biophysica Acta* 1101, 48-56, 1992.
4) Nakamura, M., Saeki, K., Takahashi, Y. Hyperproduction of Recombinant Ferredoxins in *Escherichia coli* by Coexpression of the ORF1-ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3 Gene Cluster. *J. Biochem*, 126, 10-18, 1999.

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