II.K1 SISGR: Using *In vitro* Maturation and Cell-free Evolution to Understand [Fe-Fe]hydrogenase Activation and Active Site Constraints

James Swartz[1,2] * (PI), Jon M. Kuchenreuther[1], Alyssa Bingham[1], Phillip Smith[1], Celestine Grady-Smith[3,4], Simon J. George[3], and Stephen P. Cramer[3,4]

[1] Department of Chemical Engineering, Stanford University
[2] Department of Bioengineering, Stanford University
[3] Department of Applied Science, University of California, Davis, CA
[4] Physical Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA
* Presenter's Address:
Stauffer III, Room 113
Stanford, CA 94305-5025
Phone: (650) 723-5398
E-mail: jswartz@stanford.edu

DOE Program Manager: Michael Markowitz
Phone: (301) 903-6779
E-mail: Mike.markowitz@science.doe.gov

Objectives

With this project, we seek increased understanding of the mechanisms for assembly and activation of [FeFe] hydrogenases as well as the structural role that the polypeptide scaffolding plays in forming and stabilizing this complex enzyme's active site. We are developing in vitro maturation methods to enable detailed study of the biochemical reactions required to provide precursors and to assemble the catalytic H-cluster as well as to install it into the apoenzyme. We are also searching for enzyme mutations that will reveal the role of the polypeptide structure. To accomplish these objectives and to help advance this important field of research, we are also developing improved methods for producing these complex enzymes and for evaluating various functional characteristics.

Technical Barriers

This project is designed to provide information relative to the maturation and structure function relationships of [FeFe] hydrogenases. Since these are nature's most prolific hydrogen producers, this information will facilitate the design, assembly and cost-effective production of improved biological and biomimetic catalysts for the production of hydrogen and for hydrogen based fuel cells. The project will also deliver improved methods for the evolution, evaluation, and production of improved hydrogen producing catalysts. Since the most plentiful proton source for producing hydrogen is water and oxygen is a side product, the project will also search for oxygen tolerant hydrogenase mutants and seek to understand the structure function relationships relative to oxygen tolerance.

Abstract

The first project objective is to elucidate the substrates and mechanisms for assembly and maturation of the 6Fe-6S active site of [FeFe] hydrogenases. An early *in vitro* maturation method was developed using an *E.coli* cell extract in which the three required maturases had been expressed and activated. The extract was also dialyzed to remove small molecules. We showed, for the first time, that activation of the purified apoenzyme was significantly enhanced by incubating this extract in the presence of S-adenosyl methionine, Fe(II), sulfide, and 20 amino acids. A design of experiment (DOE) investigation then showed that tyrosine was required and cysteine was beneficial for maturation. Further investigation showed that the para-hydroxyl substituent of tyrosine was required for efficient activation. An improved maturation procedure was then developed in which the three maturase proteins were expressed separately to avoid *in vivo* maturase interactions. Such interactions were thought to be important for function, and we sought to gain control over this portion of the maturation mechanism. In contrast to previous inferences, efficient apoenzyme activation was observed using the separately produced maturases. This new system enabled the production of much larger quantities of *in vitro* activated [FeFe] hydrogenase. This, in turn, enabled high resolution FTIR (Fourier transform infrared spectros-copy) analysis of the C-O and C-N stretches associated with the cyanide and carbon monoxide adducts in the 6Fe-6S active site. Isotopic labeling of tyrosine then conclusively showed that all three of the CO and both of the CN moieties in the active site are derived from tyrosine.

The second project objective is to gain better understanding of the impact of the polypeptide scaffolding on hydrogenase activity. The expression gene for the CpI [FeFe] hydrogenase from *Clostridium pasteurianum* was extensively mutated using nucleotide analogs, and isolates were identified that retained at least partial activity and also displayed increased stability during oxygen exposure. The most interesting isolate contained 13 mutations and was extensively analyzed. When two of the amino acid changes were reversed, full activity was restored. Three different mutations increased oxygen tolerance. Unexpectedly, none of these mutations are near the active site 6Fe-6S H-cluster, but four of the five mutations are close to the proximal 4Fe-4S center that either donates electrons to the active site H-cluster or receives them, depending on the direction of catalysis. These results suggest that the function of this ancillary iron-sulfur center has a stronger than expected

influence on enzyme function. During this work, we also observed that oxygen inactivation of [FeFe] hydrogenases can be substantially reversed in contrast to general belief. The reversibility also appears to be influenced by the ancillary Fe-S center. Future work will now seek to assess the function of the amino acids surrounding this ancillary center as well as those that support the active site.

During the course of these investigations, a number of methods were developed of general utility to this field of research. These include: the *in vitro* hydrogenase maturation protocols, a procedure for producing high levels of maturases and hydrogenases in *E.coli* cultures, improved methods for producing and screening hydrogenase mutants, and a new method for assessing sustained hydrogen production activity while using a reduced ferredoxin protein as the electron donor. In particular, the last method is very important as it will now allow us to screen for mutants with increased oxygen stability while they are actively making hydrogen. In addition, this method suggests the feasibility for cost-effectively producing hydrogen from biomass hydrolysates. A new research program has now been initiated to develop technology for the large scale production of hydrogen from biomass hydrolysates.

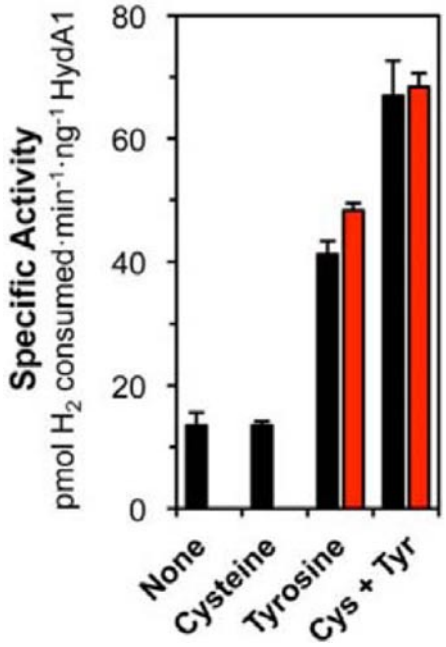

FIGURE 1.

Progress Report

Taking our lessons from the investigation of another important and complex enzyme, nitrogenase, we first sought to develop methods for the *in vitro* maturation of the [FeFe] hydrogenases. Although early work from the Peters lab at U. of Montana had achieved *in vitro* activation (although with very low yields), that system was not suitable for investigating the effects of small molecular weight substrates. Based on our success with cell-free protein synthesis of the hydrogenases (Boyer et al, 2008), we developed an *in vitro* maturation protocol that achieved substantial activation of the *C. rheinhardtii* HydA1 [FeFe] hydrogenase using cell extracts in which the three maturases, HydE, HydF, and HydG, had been expressed. These maturases were expressed from the relevant genes taken from *Shewanella oneidensis* and were produced under anaerobic conditions at 20°C to encourage proper folding. For convenience, the extracts were then prepared under aerobic conditions and were frozen for storage. The extracts were then dialyzed just before use to remove small molecules, and the maturases were reconstituted (reactivated) by anaerobic incubation with Fe(II) and S^{-2}. The apoenzyme was produced in *E.coli* cultures lacking the maturases and had no activity after purification. As expected, S-adenosyl methionine, Fe(II), and S^{-2} were necessary for activation. However, full activation was only achieved with the additional presence of a mixture of the 20 natural amino acids. A design of experiment (DOE) protocol then indicated that tyrosine was essential and cysteine was beneficial (see Figure 1) for hydrogenase activation.

A previous report had suggested that co-expression of the maturases was necessary for their activation. However, we reasoned that more information about hydrogenase maturation could be gained if the maturases could be expressed in separate *E.coli* cultures. This would then require that nearly all maturation related reactions take place during the *in vitro* activation. This protocol would also have a higher likelihood of enabling complete incorporation of isotopically labeled precursors into the H-cluster active site. Again, the apoenzyme was produced in cultures lacking the maturases and had no activity. At this point in the project, we had discovered that an *E. coli* strain with a Δ*iscR* deletion (Akhtar & Jones, 2008) produced more of the maturases as well as more apoenzyme. By mixing three extracts, each containing high levels of an individual maturase, effective apoenzyme activation was achieved. This protocol also efficiently matured the more complex CpI hydrogenase from *Clostridium pasteurianum*. After isotopically labeled tyrosine was added to the maturation reactions, the activated and purified hydrogenase samples were analyzed by FTIR. Distincitive shifts in the C-O and C-N stretches were observed as shown in Figure 2. These shifts indicated that all of the CN^- adducts in the active site H-cluster derived from the alpha carbon and amino nitrogen of tyrosine and all of the CO adducts derived from the carboxylate group of tyrosine. Recent work had shown that CO and CN could be extracted from the HydG maturase and that these species derived from tyrosine, but these recent publications had not shown that all of the adducts in the active enzyme derived from tyrosine.

The work to characterize the role of the polypeptide structure began with a mutant of the *C. pasteurianum* CpI hydrogenase that was isolated with 13 amino acid changes that conferred lower activity and improved oxygen tolerance. When the wild type amino acids were restored at positions 186 and 188, full activity was also restored. (See Figure 3)

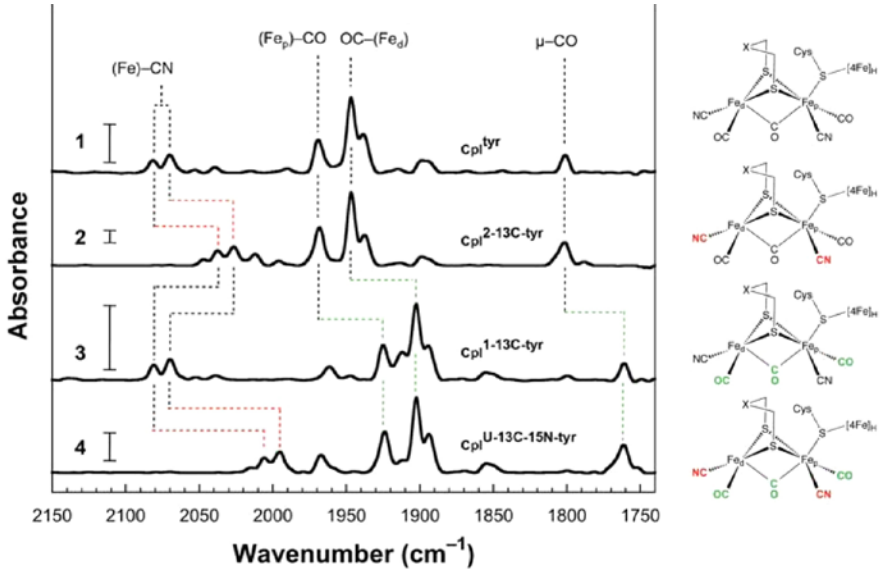

FIGURE 2.

Interestingly, these mutations were not near the active site, but rather adjacent to the electron conducting 4Fe-4S center proximal to the active site. It also turned out that the most influential mutation for conferring oxygen tolerance was at position 197, an amino acid that is adjacent to the same 4Fe-4S center. Amino acid position 160 was also influential for oxygen tolerance and was adjacent to the same cluster.

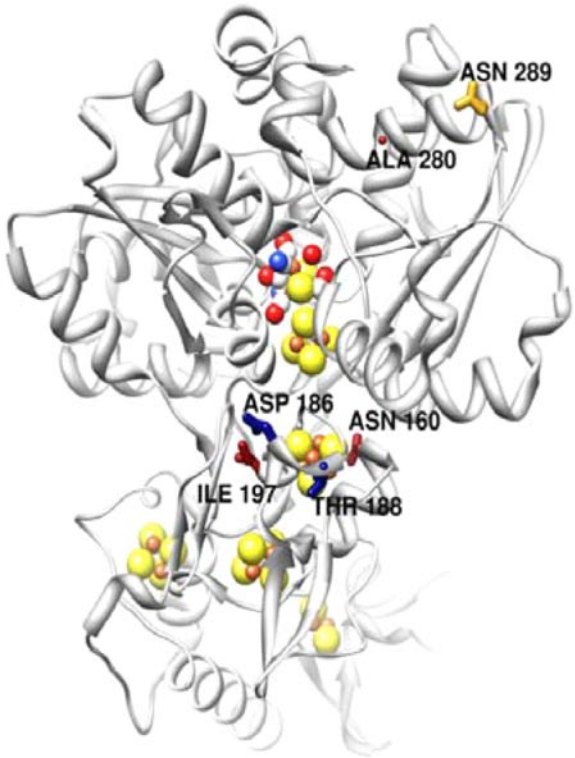

FIGURE 3.

It thus became clear that this 4Fe-4S cluster proximal to the catalytic center has strong influences on functional properties. The influences of the other amino acids surrounding this cluster will now be further characterized in addition to those surrounding the catalytic center.

Perhaps even more important than these specific discoveries is the collection of methods that have been developed. For example, paper no. 2 in our publication list describes a new production protocol that increases volumetric hydrogenase yields by 10 to 30 fold when produced by an rDNA *E.coli* culture. We have also established the ability to continually transfer electrons from NADPH to the hydrogenase to assess oxygen tolerance while the enzyme is making hydrogen. Unfortunately, this new technique showed that our oxygen tolerant mutant was only tolerant when in the resting state and not when it is producing hydrogen. This is a totally unexpected observation. It will be investigated further, but it also suggests that we need to adjust our screening assays. The new pathway from NADPH will form the basis for a high throughput screening assay that assesses oxygen tolerance for the active enzyme. This new method also forms the basis for a new pathway to convert biomass hydrolysates to hydrogen and this is now being pursued by a new project.

Future Directions

We now have accumulated a large variety of enzyme production and assessment methods and we can now enter a more active period to generate many new mutants that differ in functional characteristics. We will continue to work with our spectroscopy experts to gain a fuller understanding of the relationship between structural and functional changes and will most likely also begin a collaboration with X-ray structural experts to gain more detailed information about the impact of individual mutations. To better understand maturation, we will also work to establish catalytic maturation; i.e., the ability to catalyze multiple turnovers of the maturases when working to activate the hydrogenase.

References

1. Boyer ME …. Swartz JR. (2008) Cell-free synthesis and maturation of [FeFe] hydrogenases. Biotech. and Bioeng. **99**: 59-67.

2. Akhtar MK and Jones PR (2008) Deletion of iscR stimulates recombinant clostridial Fe-Fe hydrogenase activity and H2-accumulation in *Escherichia coli* BL21(DE3). Appl. Microbiol. and Biotechnol. **78**(5): 853-62.

Publication list (including patents) acknowledging the DOE grant or contract

1. Kuchenreuther JM, Stapleton JA, Swartz JR. (2009) Tyrosine, Cysteine and S-Adenosyl Methionine Stimulate *In Vitro* [FeFe] Hydrogenase Activation. PLoS ONE4(10): e7565.

2. Kuchenreuther JM, Grady-Smith CS, Bingham AS, George SJ, Cramer SP, Swartz JR. (2010) High-Yield Expression of Heterologous [FeFe] Hydrogenases in *E coli*. PLoS One 5(11): e15491.

3. Kuchenreuther JM, George SJ, Grady-Smith CS, Cramer SP, Swartz JR. (2011) Cell-free H-cluster synthesis and [FeFe] hydrogenase activation: all five CO and CN^- ligands derive from tyrosine. PLoS ONE (accepted, in revision)

4. Bingham AS, Smith PR, Swartz JR. (2011) Evolution of an [FeFe] hydrogenase with decreased oxygen sensitivity. International Journal of Hydrogen Energy; In Press.

5. Swartz JR, Smith PR (2010) A General Enzymatic Pathway for Hydrogen Production. Provisional Patent Application filed 9/3/2010.