# Fundamental Studies of Recombinant Hydrogenases

Michael W.W. Adams
University of Georgia
Life Sciences Bldg., Green Street
Athens, GA 30602
Phone: (706) 542-2060, Fax: (706) 542-2009
E-mail: adams@bmb.uga.edu

DOE Program Officer: Robert Stack
E-mail: Robert.Stack@science.doe.gov

Subcontractors: n/a

## Objectives

The Hydrogen Fuel Initiative Workshop report (www.science.doe.gov/bes/hydrogen.pdf) sponsored by DOE stated that a fundamental understanding is needed of the structure and chemical mechanism of the enzyme complexes, known as hydrogenases, that support hydrogen generation, and that we need to understand how these catalysts are assembled with their cofactors into integrated systems. The proposed research specifically addresses the issues of understanding the assembly and organization of hydrogenases. This is the first step to ultimately reducing their size and complexity and of determining structure/function relationships, including energy conservation via charge separation across membranes.

## Technical Barriers

Remarkably, in spite of the large amount of research that has been carried out on hydrogenases, it is not possible to readily manipulate or engineer the enzyme using molecular biology approaches since a recombinant form produced in suitable amounts in a suitable host is not available. Such resources are essential if we are to understand what constitutes a "minimal" hydrogenase, and to design such catalysts with desired properties, such as resistance to oxygen, extreme stability and specificity for a given electron donor. However, this is very challenging from a technical perspective as numerous gene products are required to synthesize the catalytic nickel-iron (NiFe) catalytic site of the protein and to assemble the mature, active hydrogenase enzyme.

## Abstract

As a model system we are using the NADP-dependent, cytoplasmic NiFe-hydrogenase I (SHI) of *Pyrococcus furiosus,* a hyperthermophile that grows optimally at 100°C. We made two breakthrough discoveries during the funding period. First, we have generated the catalytically-active form of the heterotetrameric SHI by heterologous gene expression in the mesophilic bacterium, *Escherichia coli.* Second, a genetic system is now available in *P. furiosus* and this allows us to generate modified forms of the SHI by homologous gene expression. Specifically, we have overexpressed SHI in *P. furiosus* such that the organism produces an order of magnitude more of the native enzyme, increasing the amount purified from native biomass by up to 50-fold. We have also taken the first step toward generating a 'minimal' hydrogenase by homologous expression of an affinity-tagged heterodimeric form of the heterotetrameric enzyme and by so doing we have also changed its electron carrier specificity. In contrast to the native form, the heterodimer does not use NADP/H as an electron carrier and directly interacts with the pyruvate-oxidizing enzyme, pyruvate ferredoxin oxidoreductase, thereby enabling direct hydrogen production from pyruvate by an as yet unknown mechanism.

## Progress Report

### 1. Production of the Native Tetrameric Form of *P. furiosus* hydrogenase I (SHI) in *E. coli.*

Hydrogenases are extremely complex, air-sensitive enzymes that achieve catalysis using a binuclear nickel-iron cluster that contain cyanide and carbon monoxide ligands to the iron atom (see **Figure 1**). This cluster is covalently bound to the protein and receives electrons for proton reduction via multiple iron-sulfur (FeS) clusters. A very complicated maturation process is required to synthesize the [NiFe]-catalytic center and insert it into the apoenzyme (see **Figure 1**). We successfully produced in *Escherichia coli* the recombinant form of *P. furiosus* SHI using novel expression vectors for the co-expression of thirteen *P. furiosus* genes (four structural genes encoding the hydrogenase and nine encoding maturation proteins). Remarkably, the native *E. coli* maturation machinery will also generate a functional *P. furiosus* hydrogenase when provided with only the genes encoding the hydrogenase subunits and a single protease (frxA) from *P. furiosus*. Another novel feature is that its expression was induced by anaerobic conditions, whereby *E. coli* was grown aerobically and production of recombinant hydrogenase was achieved by simply changing the gas feed from air to an inert gas ($N_2$). The recombinant enzyme was purified and shown to be functionally similar to the native enzyme purified from *P. furiosus*, including its high stability towards oxygen ($t_{1/2} > 20$ hr in air) and the use of NADP(H) as an electron carrier. An enzyme that evolves hydrogen from NADPH holds promise for large-scale harvesting of molecular hydrogen from renewable biomass, and the methodology to produce the key hydrogen-producing enzyme has now been established.

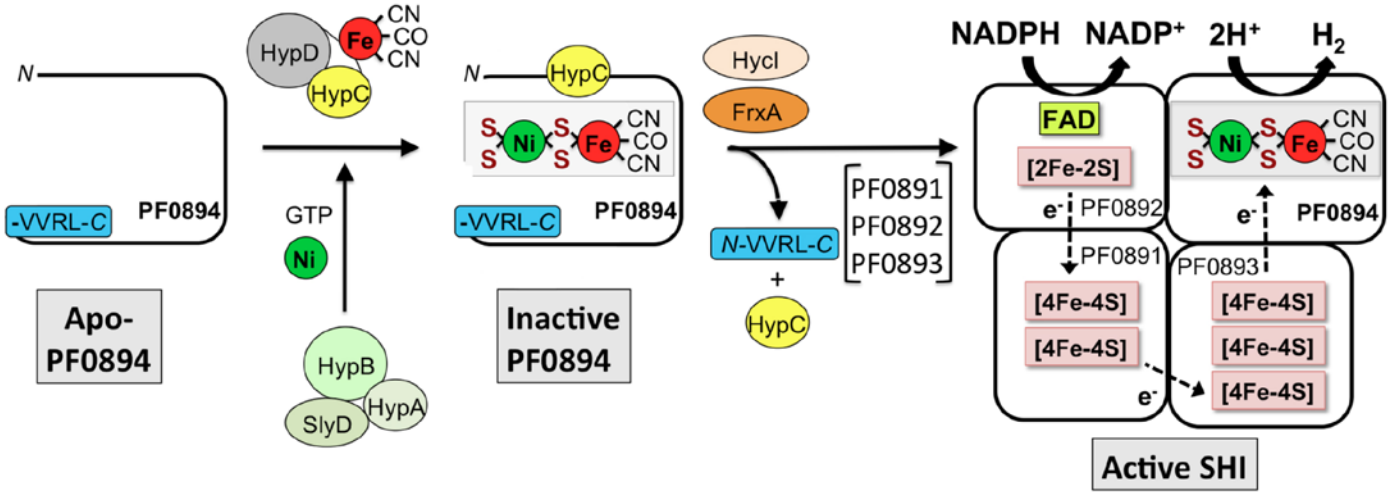


**FIGURE 1.** Biosynthesis of *P. furiosus* hydrogenase I (SHI) in *E. coli.*

**2. Over-Production of the Native Tetrameric Form of *P. furiosus* Hydrogenase I (SHI) in *P. furiosus.***

A major breakthrough in the past year has been the development of a genetics system in *P. furiosus* in another on-going project in the PI's laboratory. We are utilizing this to develop expression systems for the hydrogenase I. This gives us the capability for both heterologous (in *E. coli*) and homologous (in *P. furiosus*) systems to engineer *P. furiosus* hydrogenases. Our first objective was to obtain a *P. furiosus* strain in which SHI was significantly over-expressed and where the protein contained an affinity tag for rapid purification. To achieve this we utilized a parental strain that had a deletion in a gene (*pyrF*) required for uracil biosynthesis in *P. furiosus.* This requires uracil for growth and is resistance to the uracil biosynthetic pathway inhibitor, 5-fluoroorotate (5-FOA). A 'knock-in' cassette was constructed containing the upstream (UFR) and downstream (DFR) flanking regions of the promoter region for SHI ($P_{SHI}$), together with *pyrF* (driven by the promoter for glutamate dehydrogenase, $P_{GDH}$), a terminator sequence (T1) and replacing $P_{SHI}$ with $P_{SLP}$, the promoter for the S-layer protein (PF1399), and the strep-II tag (8 amino-acids) at the N-terminus of the PF0891 subunit of SHI. $P_{SLP}$ is one of the most highly expressed genes in *P. furiosus* based on our DNA microarray data.

The specific hydrogenase activity of cytoplasmic extracts of wild type *P. furiosus* cells and the Δ*pyrF* strain were 0.77 ± 0.5 units/mg. The two strains in which SHI was over-expressed (OE-SHI) by $P_{SLP}$ showed no obvious phenotype but their specific activities were 8.2 ± 0.2 units/mg, representing an increase of more than an order of magnitude in hydrogenase activity. The dramatic overexpression of SHI protein in the OE-SHI strains was confirmed by immuoanalysis using antibodies to PF0894 (the catalytic subunit of SHI). Given the extremely complex maturation process that is involved in synthesizing the hydrogenase, the results show that the wild-type level of the maturation machinery is able to process >10-fold more SHI than it usually does. This was somewhat surprising since no attempt was made to up-regulate these processing genes. Unfortunately, the Strep-II tag on PF0891 appeared to be partially buried as OE-SHI bound weakly to the strep-tactin column. Nevertheless, while five chromatography steps are normally needed to purify native SHI yielding 2-10 mg /500 g of cells, the OE-SHI strain yielded 11 mg/50 g of cells. The native and OE-forms had similar thermal stabilities and oxygen sensitivities.

**3. Over-Production of a Heterodimeric form of *P. furiosus* Hydrogenase I (SHI) in *P. furiosus.***

As a first step towards making a 'minimal' hydrogenase, we produced the heterodimeric form of SHI containing only the large catalytic (LSU; PF0894) and the small FeS-cluster containing subunit (SSU, PF0894), as shown in **Figure 2**. The SSU contained an N-terminal $His_9$-tag and expression was driven by the $P_{SLP}$ promoter. Recombinant Pf cells had no significant phenotype and a total of ~6 mg of the pure protein (148 U/mg) was obtained from the Ni-NTA column using 50 g of cells. The OE-dimer was less thermostable ($t_{1/2}$ 1 hr at 90°C) and more oxygen sensitive ($t_{1/2}$ 1 hr in air) than native heterotetrameric form of SHI. The dimer did not evolve $H_2$ from NADPH (since it lacked FAD-containing PF0892) nor did it evolve $H_2$ from reduced ferredoxin of *P. furiosus.* However, in contrast to the native form, the heterodimer directly interacted with the pyruvate-oxidizing enzyme, pyruvate ferredoxin oxidoreductase (POR) from *P. furiosus,* thereby enabling direct hydrogen production from pyruvate. It therefore appears that the novel heterodimeric hydrogenase is able to accept electrons by a direct enzyme-enzyme (hydrogenase-POR) interaction although the mechanism is unknown.

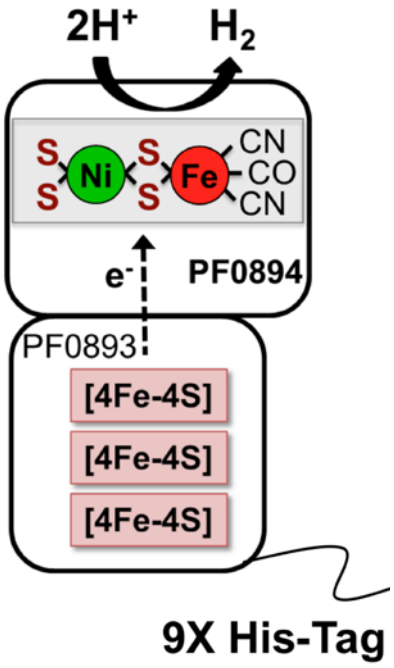


**FIGURE 2.** Structure of the heterodimeric form of SHI.

## Future Directions

The longer term objectives of our research are to capitalize on the breakthroughs made in the current funding period and to a) optimize production of tagged recombinant forms of the cytoplasmic hydrogenase of *P. furiosus*, b) to obtain soluble and membrane-bound tagged recombinant forms of the ferredoxin-dependent, membrane-bound hydrogenase that is also present in *P. furiosus,* an enzyme that we have shown simultaneously evolves $H_2$ and pumps protons, c) to design 'minimal' hydrogenases with tailored catalytic activity, oxygen sensitivity and electron donor specificity, and d) to design membrane-bound hydrogenases in artificial membranes that generate ion gradients and evolve hydrogen. Such enzymes have potential utility in various biotechnological applications.

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

**1.** Sun, J., Hopkins, R.C., Jenney, F.E., Jr., McTernan, P. and Adams, M.W.W. (2010) Heterologous Expression and Maturation of an NADP-Dependent [NiFe]-Hydrogenase: A Key Enzyme in Biofuel Production. *PLoS ONE* 5:e10526, 1-11

**2.** Baker, S.E., Hopkins, R.C., Blanchette, C.D., Walsworth, V.L., Sumbad, R., Fischer, N.O., Kuhn, E.A., Coleman, M., Chromy, B.A., Létant, S.E., Hoeprich, P.D., Adams, M.W.W., and Henderson, P.T. (2009) "Hydrogen production by a hyperthermophilic membrane-bound hydrogenase in soluble nanolipoprotein particles" *J. Am. Chem. Soc.* 131, 7508-9

**3.** Ye, X., Wang, Y., Hopkins, R.C., Adams, M.W.W., Evans, B.R., Mielenz, J.R. and Zhang, Y.H. (2009) "Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails" *Chem. Sus. Chem.* 2, 149-152

**4.** Jenney, F.E., Jr. and Adams, M.W.W. (2008) "Hydrogenases of the model hyperthermophiles" *Ann. N.Y. Acad. Sci.* 1125, 252-266

**5.** Chou, C.-J, Jenney, F.E., Jr., Adams, M.W.W. and Kelly, R.M. (2008) "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels" *Metabolic Engineer.* 10, 394-404

**6.** Adams, M.W.W., Hopkins, R.C., Jenney, F.E., and Sun, J. (2007) Hydrogenase Polypeptides and Methods of Use, US Provisional Patent Application (#61/005,383; filed 12/05/07)

**7.** Schut, G., Bridger, S.L. and Adams, M.W.W. (2007) Insights into the metabolism of elemental sulfur by the hyperthermophilic archaeon *Pyrococcus furiosus*: characterization of a coenzyme A-dependent NAD(P)H sulfur oxidoreductase. *J. Bacteriol.* 189, 4431-4441

**8.** Zhang, Y.H.P., Evans, B.R., Mielenz, J.R., Hopkins, R.C. and Adams, M.W.W. (2007) High yield hydrogen production from starch and water by a synthetic enzyme pathway. *PLoS One* 5, e456 (1-6)