II.K.5 Fundamental Studies of Recombinant Hydrogenases

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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. This is very challenging from a technical perspective as numerous gene products, including some not yet characterized, are required to synthesize the hydrogenase enzyme. The primary goal of the proposed research is to produce in *E. coli* catalytically-active, recombinant forms of a model NiFe-type hydrogenase, in this case from a hyperthermophilic organism that grows at 100°C.

Abstract

In this funding period we made the breakthrough discovery that a catalytically-active, recombinant form of the cytoplasmic, NADPH-dependent, NiFe-hydrogenase of *Pyrococcus furiosus,* a hyperthermophile that grows optimally at 100°C, could be produced by heterologous gene expression in the mesophilic bacterium, *Escherichia coli* (a patent application has been filed). In addition, a genetic system is now available in *P. furiosus* and this allows us to generate modified forms of the hydrogenase by homologous gene expression. We also used native *P. furiosus* hydrogenase to construct, in a collaborative study, an *in vitro* metabolic pathway not found in any organism that catalyzes the complete conversion of starch to H_2 and CO_2. Current research is focused on optimizing the production of, and characterizing the recombinant forms of various recombinant forms of *P. furiosus* hydrogenase and engineering minimal forms of the enzyme through both heterologous and homologous gene expression.

Progress Report

In microorganisms, the molecular machine responsible for the biological uptake and evolution of H_2 is an enzyme known as hydrogenase. Hydrogenase catalyzes the simplest of chemical reactions, the interconversion of the neutral molecule H_2 and its elementary constituents, two protons and two electrons, according to the following equation: $2H^+ + 2e^- \leftrightarrow H_2$. Ironically, however, while the reaction that they catalyze is simple, hydrogenases are exceedingly complex proteins and typically are extremely sensitive to air (oxygen). This complexity has to date precluded the facile production of a recombinant form of the major class of hydrogenase, the so-called 'nickel-iron' (NiFe) type. Through the work described here, we have generated in *E. coli* a soluble, recombinant thermostable NiFe-hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus,* an organism that grows optimally at 100°C. We also demonstrate that this type of hydrogenase can be used in a prototype hydrogen production system. The availability of a recombinant enzyme also provides the means to obtain new versions of the hydrogenase that are more active and/or more simple and/or use different electron donors and acceptors.

a. Production of Active Recombinant *Pyrococcus furiosus* Hydrogenase I (Pf SHI) in *E. coli*

In order to produce catalytically-active, recombinant forms of *P. furiosus* (Pf) cytoplasmic hydrogenase I (SHI), *E. coli* must be grown anaerobically. For reasons that are still not clear, the IPTG-induced promoter systems such as T7 do not function efficiently when *E. coli* is grown anaerobically. Our initial goal was to

establish an anaerobic expression system in *E. coli,* and this has been accomplished using the P-ana system. The focus of the recombinant work has been the tetrameric holoenzyme form (PF0891-PF0894) of Pf SHI, which includes the catalytic NiFe-containing subunit, PF0894. At least eight maturation genes (HypCDAB, HypEF, HycI and SlyD) are thought to be required in Pf to generate the active form of SHI. In addition, a helper plasmid, pRIL, is required because of the differences in codon usage between Pf and *E. coli*. Together with the structural genes and the pRIL system, as many as 13 genes might have to be co-expressed in *E. coli* if active recombinant SHI is to be obtained. Significant effort has therefore been put into the design and construction of multiple expression vectors for producing recombinant Pf proteins in *E. coli.* To do this we capitalized on the versatility of the Gateway™ vector system (Invitrogen), which allows rapid recombinational cloning independent of restriction enzymes. We have constructed four different vectors such that they have compatible origins of replication but different antibiotic selection and allow the simultaneous expression of up to 13 Pf genes controlled by the anaerobic promoter system.

Transcriptional analyses have shown that the target Pf genes cloned into the four different vectors are all expressed, even when as many as five genes are cloned on one vector. Protein expression from many of these genes is sufficiently high to be detectable by SDS-PAGE and by antibodies to PF0894. In most cases the identity of the recombinant proteins have been confirmed by MS analyses after tryptic digestion. Attempts to express recombinant *Pf* hydrogenase have been assessed under completely anaerobic conditions using the P-ana system. The host for such expression is a mutant strain of *E. coli* (MW1001) that is lacking the structural genes for all three native *E. coli* hydrogenases. This strain provided the hydrogenase-deficient background for detection of recombinant Pf hydrogenase activity. Assays for the Pf enzyme are by H_2 evolution using reduced methyl viologen as the electron donor at 80°C.

The recombinant Pf hydrogenase produced in concert with HypCDAB, HypEF, HycI and SlyD is active at 80°C and located in the cytoplasmic fraction. Moreover, this soluble form is thermostable, showing no loss of activity after 30 min at 80°C. Indeed, there is 50% increase in total activity after the heat treatment, and the specific activity increases 8-fold, as many of the *E. coli* proteins precipitate upon heating. Given the breakthrough nature of this first successful heterologous expression of a recombinant hyperthermophilic NiFe hydrogenase in *E. coli*, a provisional patent application has been filed (Adams et al., 2007). The recombinant Pf enzyme forms the basis for our current research in which we are optimizing production of the recombinant form and are producing 'minimal' forms. In addition, through genome analyses, we have identified a putative ninth gene that is potentially involved in the processing of Pf SHI. We are currently investigating the potential for this enzyme to increase the yields of the recombinant hydrogenase. We are also studying the effects of growth media and the levels of expression of the genes encoding the various processing proteins on the yield of recombinant SHI.

b. Use of Pf SHI for starch to hydrogen conversion

In addition to its high thermal stability, Pf SHI is of great potential utility because it has the ability to generate H_2 by oxidizing NADPH, a rare property among the hydrogenases that have been characterized to date. This is particularly relevant in H_2 production systems where carbohydrates are oxidized to generate NADPH, which can be directly converted to H_2 by SHI. Using this system, we previously demonstrated the production of H_2 from glucose in an *in vitro* cell-free system using two purified enzymes, which include Pf SHI. In collaboration with Dr. P. Zhang (Virginia Tech) and Dr. J. Mielenz (Oak Ridge National Laboratory), this work was extended to the conversion of starch to H_2 by an *in vitro* cell-free system made up of thirteen different enzymes (Zhang et al., 2007). Twelve of the enzymes are used to oxidize starch and generate CO_2 and NADPH, while the thirteenth, Pf SHI, oxidizes NADPH and produces H_2. The overall pathway, which does not exist in any microorganism, is shown in Figure 1.

A key step is direct phosphorylation of the carbohydrate (Figure 1), which utilizes energy stored in the glucosidic bonds of starch to produce the activated phosphorylated monosaccharide without ATP consumption. Glucose-6-phosphate (G6P) is then completely oxidized to CO_2 by 10 enzymes of the pentose phosphate pathway and NADPH that is produced is used by Pf SHI to generate H_2. Thermodynamic analysis shows that the overall reaction is spontaneous ($\Delta G^o = -34.8$ kJ/mol). Proof of principle of the reaction was demonstrated at 30°C using Pf SHI and a total twelve enzymes from mesophilic (commercial) sources. Increasing the activity of Pf SHI at "low" temperatures is one of the goals of our future research. Starch has a very high energy and volume storage density for H_2 (14.8 H_2-mass %, 104 kg H_2/m^3), which exceed those of current synthetic materials and exceed DOE storage goals. Solid starch would address many issue of the H_2 economy infrastructure.

So far in the synthetic pathway we have used Pf SHI purified from Pf biomass but future development of the starch to H_2 process would be greatly facilitated by the availability of a recombinant form of SHI. The goals of the current research are to produce the recombinant enzyme a) in large quantities, b) with an optional tag to enable facile purification and/or immobilization, and c) in mutant forms that are more active at low temperature and/or use different electron carriers.

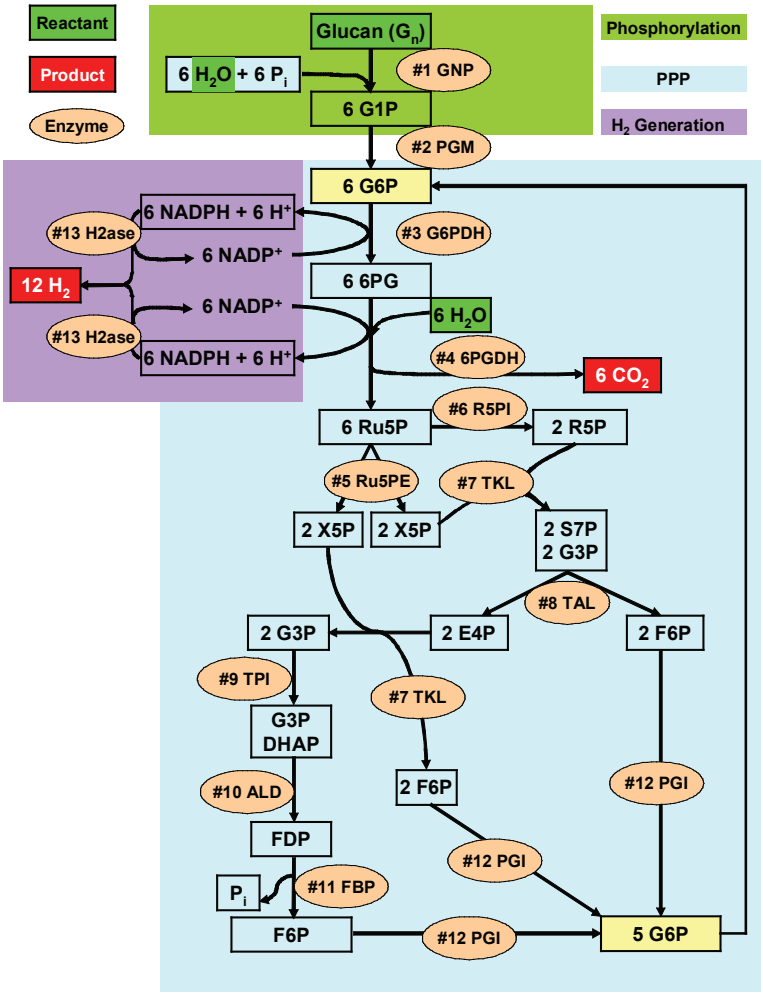

FIGURE 1. The synthetic metabolic pathway for complete conversion of starch and water to H_2 and CO_2 using Pf SHI. The color coding represents the phosphorylation step (green), the pentose phosphate pathway (PPP, blue) and the H_2 production step catalyzed by SHI (purple). The abbreviations for the enzymes and the intermediates are given in Zhang *et al.* (2007).

c. Regulation of expression of the three hydrogenases (SHI, SHII and MBH) of P. furiosus

Research in the PI's laboratory has recently led to the development of a genetic system in Pf (not funded by this DOE award; Westpheling and Adams, unpublished). This now enables us to use Pf to generate various recombinant forms of SHI with desired properties through selection and evolutionary approaches.
A prerequisite for utilizing Pf is an understanding of how it regulates the expression of the genes encoding SHI, as well as those of a second cytoplasmic enzyme, SHII, and of the the H_2-evolving membrane-bound hydrogenase, MBH. We have now shown that when elemental sulfur (S°) is added to a growing Pf culture, a primary response occurs within 10 min whereby H_2 production ceases and H_2S is produced. This response involves the dramatic down-regulation of the expression of the 14-gene cluster encoding MBH and of the 8 genes encoding SHI and SHII (Schut et al., 2007). In collaborative studies with Dr. R. Scott (University of Georgia), it was shown using DNA-baiting experiments that the transcriptional regulator involved in the primary S° response is SurR (PF0095: Lipscomb and Scott, unpublished). Various gene(s) encoding SHI and derivatives are currently being constructed and these will be used to generate minimal forms as well as to 'overexpress' SHI in Pf.

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, d) to design membrane-bound hydrogenases in artificial membranes that generate ion gradients and evolve hydrogen, and e) to develop robotic protocols for high-throughput hydrogenase activity screening and use them to obtain novel 'designer' hydrogenases by evolutionary approaches. Such enzymes have potential utility in various biotechnological applications.

Publications (including patents) acknowledging the grant or contract

1. 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)

2. 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

3. 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)