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FINAL TECHNICAL REPORT (8/1/94 to 7/31/97)

Catalytic Mechanism of Hydrogenase from *Azotobacter vinelandii*

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Introduction

Our current DOE-sponsored project is focused on investigations of the catalytic mechanism of the hydrogenase found in the aerobic, N_2-fixing microorganism *Azotobacter vinelandii*.. The following pages summarize our progress during the first two years of the current project and include the anticipated course of the research for the remaining year of the current project. Because the current proposal represents a change in direction, we also include a brief progress report of prior DOE-sponsored research dealing with hydrogenases.

Hydrogenases are enzymes which catalyze reactions involving dihydrogen. They serve integral roles in a number of microbial metabolic pathways. As one example, hydrogenases oxidize the H_2 generated by nitrogenase in biological N_2 fixation. This "recycling" of H_2 leads to an increased efficiency of N_2 fixation. This hydrogenase is representative of the class of Ni- and Fe-containing heterodimeric hydrogenases. DOE's sponsorship of our work on this and related hydrogenases began in 1983. Our initial focus was on the NiFe hydrogenase from *Bradyrhizobium japonicum* bacteroids isolated from soybean nodules in addition to the hydrogenase from *A. vinelandii*.. We developed protocols for purifying these hydrogenases to homogeneity, examined the metal content, determined the mechanism of inhibition and inactivation by several inhibitors, and determined the N-terminal amino acid sequences of each subunit which led to isolate the genes. We demonstrated that acetylene is a slow-binding, tight-binding inhibitor which is competitive with H_2. Our demonstration that acetylene binds to the large subunit provided the first biochemical evidence that the site of H_2 activation was on the large subunit. More recently, we have focused entirely on the hydrogenase from *A. vinelandii*. This free-living bacterium can be grown in large quantities in the laboratory and produces a hydrogenase that is typical of several NiFe hydrogenases. With this bacterium, we developed methods for site-specific mutagenesis of the hydrogenase gene to produce amino acid substitutions in various residues. Through this approach, we have demonstrated the importance of the small subunit in catalysis, and have determined the importance to catalysis of several residues in the large subunit.

To date, our DOE-sponsored research has resulted in 25 peer-reviewed manuscripts with contributions to Biochemistry, Journal of Biological Chemistry, Journal of Bacteriology, Biochimica Biophysica Acta, and other journals. An additional 5 manuscripts were published as book chapters in conference proceedings volumes or topical books. Numerous presentations (invited talks, poster sessions) were made at scientific conferences.

Our work in the current project period has followed closely the objectives of the grant proposal. The work to date has resulted in the following publications:

McTavish, H., L.A. Sayavedra-Soto, and D. J. Arp. Substitution of *Azotobacter vinelandii* Hydrogenase Small-Subunit Cysteines by Serines Can Create Insensitivity to Inhibition by O_2 and Preferentially Damages H_2 Oxidation over H_2 Evolution. J. Bacteriol. 177: 3960-3964 (1995).

McTavish, H., L.A. Sayavedra-Soto, and D.J. Arp. Comparison of isotope exchange, H_2 evolution, and H_2 oxidation activities of *Azotobacter vinelandii* hydrogenase. Biochim. Biophys. Acta. In press (1996).

In addition, a manuscript detailing the effects of substitutions of amino acid residues in the large subunit on activity and Ni processing is in preparation. Our progress towards each of the four objectives of the grant follows:

Objective 1. Using site-directed mutagenesis of *hoxK* and *hoxG* (which code for the small and large subunits of hydrogenase respectively), prepare proteins with substitutions in specific amino acids which are likely to ligate metals or which are found in conserved motifs.

Among different microorganisms the genes for the dimeric NiFe hydrogenases are similar. The highest degree of similarity is in the spatial arrangement of the Cys of both subunits and in the His residues of the large subunit. In the last proposal and during the beginning of the present proposal we produced mutants in all Cys residues hypothesized as serving as ligands for the metal cofactors (for the FeS clusters and for Ni). The amino acid changes and the phenotype of the mutant strains are summarized in Table 1. The Cys changed are homologous to the residues serving as ligands for the metal cofactors recently assigned in the crystal structure of the hydrogenase of *Desulfovibrio gigas* (3). We also have produced several mutants containing changes in the conserved His residues of the large subunit, which apparently and according to the crystal structure of *D. gigas* hydrogenase, do not have a role binding the metal cofactors. These mutants we have found to have altered hydrogenase activity as well.

In the hydrogenase of *A. vinelandii* all the Cys residues to which the binding of the FeS clusters binding is attributed were individually changed to Ser and presented altered hydrogenase activities (Table 1 and (1)). We have further characterized some of these mutants in the small subunit. The results were published in the Journal of Bacteriology (see above). The mutants chosen were at the Cys 294→S, Cys297→S and Cys64,65→S in the hydrogenase small subunit. Partial hydrogenase activity was retained in mutants Cys 294→S, Cys297→S and Cys64,65→S. The H_2 oxidation activity was affected to a larger extent than the H_2 evolution activity. The ratio of H_2 oxidation-to-H_2 evolution in whole cells of the mutants was 1.5 compared to 45 observed in the wild-type hydrogenase. The results suggest that the cluster 3Fe4S can be altered or possibly eliminated and still preserve some hydrogenase activity. Membranes with K294C→S or K297C→S hydrogenase were uninhibited by O_2 in H_2 oxidation and uninhibited by H_2 during H_2 evolution. Wild-type membranes and membranes with Cys64,65C→S hydrogenase were both sensitive to these inhibitors. The data suggest that the 3Fe4S cluster is involved in the reversible inhibition of hydrogenase activity by O_2 and H_2. In this paper a modification for the aerobic purification of hydrogenase with yields comparable to the anaerobic purification of hydrogenase was also reported. Prior to this, aerobic purification of hydrogenase yielded only a fraction of the activity of that obtained under the anaerobic procedure.

Mutations to the large subunit of hydrogenase included Cys431, 434, 937 and 940 and were changed to Ser or randomly to other amino acids. The change Cys431→Ser in hydrogenase retained H_2 oxidation and H_2 evolution activities. Changes to the Cys434, 937, and 940 resulted in loss of hydrogenase oxidation and evolution activities. Ni incorporation experiment with ^{63}Ni were performed to determine if Ni was present in the hydrogenase of these mutants. The mutant Cys431→Ser presented Ni levels comparable to the wild-type strain. Cys431→Ser also presented a higher K_M for H_2. Hydrogen evolution in this mutant was approximately that of the wild-type. Ni was not incorporated in the hydrogenase of the mutants Cys434, 937, and 940. Hydrogenase in the wild-type is processed at the C terminal of HoxG and is induced by Ni. We have also determined if Ni incorporation into HoxG was essential for the processing of the large subunit in the mutant strains. The hypothesis is that Ni may exert a conformational change

which allows a protease to process the C-terminal of HoxG. The hydrogenase in the HoxG mutants were processed. However, the incorporation of Ni did not occur in the mutants of HoxG that show no activity. The addition Ni during the assay or incubations with Ni prior to the assay in concentration as high as 0.5 M did not restored H_2 oxidation in the mutants of Cys434→Ser, Cys937→Thr, and Cys940→Arg. Mutant Cys431→Ser apparently is capable of partially binding Ni and thus retains a decreased activity. This mutant lost activity readily upon changes in pH. Hydrogenase mutants Cys937→Thr and Cys940→Arg apparently accelerate the degradation of hydrogenase HoxG subunit (Fig. 1). These mutants have presented a challenge for the purification of the hydrogenase. The data are being prepared for submission to the Journal of Bacteriology.

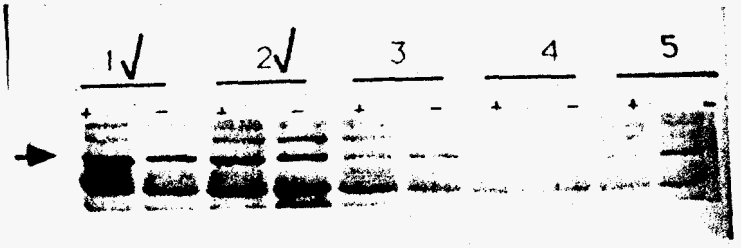

Fig. 1. Processing of hydrogenase's large subunit HoxG. Lanes 1: wild-type; 2, Cys431→Ser; 3, Cys434→Ser; 4, Cys937→Thr; 5, Cys940→Arg. The bold arrow points to HoxG. Equal amounts of total protein were in the samples used in the blot. The lower levels of HoxG can be observed in lanes 4 and 5. The signs "+" in the lanes are samples incubated in the presence of Ni. The signs "-" in the lanes are cultures to which nitrilotriacetic acid was added as Ni quelator. The large subunit was detected by immunoblotting with a rabbit antibody for the large subunit of the holoenzyme of hydrogenase. ^{63}Ni was detected by scintillation counting as is indicated by ✓ in the sample with added Ni.

Objective 2. Select hydrogenase mutants with specific phenotypes following random mutagenesis directed at *hoxK and hoxG.*

Random mutagenesis was performed in regions of high homology among hydrogenases. The areas chosen for random mutagenesis were those that differentiate hydrogenases with predominant H_2 oxidation activity from hydrogenases with predominant H_2 evolution activity. Mutagenesis was carried with oligonucleotides of about 80 bases in length with at least one random base change. Mutants were detected by colony hybridization in the HoxKG- mutant and tested for altered hydrogenase activity. After screening approximately 400 putative mutants that presented slightly diminished hydrogenase activity 10 were selected for further characterization. The mutants generated selected by this approach will be studied to determine if the mutations induced changes in the hydrogenase activities of oxidation, reduction and isotope exchange.

Objective 3. Purification and characterization of proteins with amino acid substitutions.

Purification of the hydrogenase from the mutants has presented a challenge. Apparently the rate in which the enzyme is degraded proceeds at a faster rate in some of the mutants than in the wild-type (Fig. 1). The monitoring of the levels of enzyme and the detection of the enzyme during purification are performed by immunoblotting (since many of the mutants do not present activities high enough to facilitate monitoring of the purification). Although the purification of the wild-type enzyme is well established in the laboratory, the mutant strains present subtle differences that have complicated the purification of hydrogenase from the mutants. Nevertheless we have gathered 200 μg of almost pure hydrogenase from each of the mutants Cys65→ S, Cys64,65→ S, Cys431→S, and Cys434→S. These mutants will be analyzed for changes in the metal cofactors.

Objective 4. Purify and characterize the *hoxZ* gene product or the complex consisting of hydrogenase and the *hoxZ* gene product.

In order to determine the role of HoxZ during hydrogenase activity we proposed to purify HoxZ to perform in vitro studies. We compared the wild-type strain with the HoxZ mutant (231-base pair fragment deleted in the gene for HoxZ (2)). Comparisons using polyacrylamide-SDS and two dimensional gel electrophoresis did not yield a discernible difference. Preparative steps before electrophoresis using detergents and ion exchange or size exclusion chromatography also did not result in an identifiable difference. An alternative protocol for the purification of HoxZ was the overexpression of *hoxZ* in *E. coli*. The overexpression of *hoxZ* will allow the production of an antibody specific for HoxZ to purify the HoxZ protein form *A. vinelandii* cell extracts by immunoaffinity chromatography. Overexpression in *E. coli* was carried out by cloning fragments containing *hoxZ* with the *A. vinelandii* ribosome binding sites (rbs) into pTrc99A (Pharmacia) and T7-based promoter expression vectors. These overexpression experiments did not yield an overexpressed product apparently due to the inability of *E. coli* to use *A. vinelandii* rbs sequences. An alternative protocol for overexpression of HoxZ was the cloning of a PCR amplified fragment into the pET-15 vector (Novagen) which inserts a His•Tag that allows purification in a Ni-affinity column. The strains containing the pET construct are being grown to gather enough material for protein purification. The identity of the protein will be corroborated by determining the N-terminus amino acid sequence.

The characterization of the protein's heme moieties is being approached by site directed mutagenesis of the putative His ligands to the heme(s). Four His in or near the transmembrane sequences of homologous proteins are conserved in several species containing membrane-bound NiFe hydrogenases. The codons for His will be changed to codons for Ala. The change should result in the loss of the ligand to the heme. Several putative plasmids in *E. coli* in which the desired mutation was induced are being screened. The mutation will be transferred to the chromosome by double recombination as described (1). The mutants will be detected by altered hydrogenase activity. Heme content and the altered hydrogenase activities will be determined

Developments in other directions.

Overexpression of hydrogenase. A suggestion by a reviewer of this proposal was that of testing for overexpression of hydrogenase in *A. vinelandii* Towards this we constructed a *A. vinelandii* mutant in which the nif promoter of *A. vinelandii* nitrogenase was inserted in the operon of hydrogenase. The insertion of the nif promoter was performed by creating a restriction site at the beginning of the hydrogenase structural genes. The nif promoter was obtained by PCR and cloned into the PCRII vector (Invitrogen). The insertion was transferred to the chromosome of *A. vinelandii* by double recombination in the mutant HoxKG$^-$ mutant. The mutation resulted in recovery of hydrogenase activity in the HoxKG$^-$ mutant strain, unfortunately the construct did not enhance the hydrogenase activity nor hydrogenase protein production. Apparently the nif promoter induced the protein at equal levels as the indigenous promoter. It is also likely that the expression of hydrogenase is controlled by other factors in addition to the induction of its promoter.

Comparison of hydrogenase activities in the wild-type strain. We also made comparisons of isotope exchange, H_2 evolution, and H_2 oxidation activities of hydrogenase wild-type in *A. vinelandii*. These results are being published in Biochimica et Biophysica Acta. Aerobically purified hydrogenase was used in this study. The energy of activation for oxidation and evolution activities were determined. E_A was 10 kcal/mole for H_2 oxidation and 22 kcal/mole for evolution. The velocity of H_2 evolution and isotope exchange were equal suggesting that both reactions share the same-limiting step. D_2 and H_2 inhibited H_2 evolution, but did not inhibit isotope exchange. We conclude that H_2 and D_2 evolution by competing with

H^+ for the site of the reduced enzyme. The k_M for D_2 in isotope exchange is 40 times greater than its K_M in D_2 oxidation.

References:

1. **Sayavedra-Soto, L. A. and D. J. Arp** 1993. In *Azotobacter vinelandii* hydrogenase, substitution of serine for the cysteine residues as positions 62, 65, 289, and 292 in the small (HoxK) subunit affects H_2 oxidation. Journal of Bacteriology **175:** 3414-3421.
2. **Sayavedra-Soto, L. A., N. H. Hommes, and D. J. Arp** 1994. Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. J. Bacteriol. **176:** 504-510.
3. **Volbeda, A., M.-H. Charon, C. Piras, E. C. Hatchikian, M. Frey, and J. C. Fontecilla-Camps** 1995. Crystal; structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. Nature **373:** 580-587.

DISCLAIMER

Table 1. Changes to the putative amino acids binding the metal cofactors in hydrogenase of *A. vinelandii* and the resulting phenotype and strains with gene insertions and deletions in the HoxKG operon.

	Amino acid change	H_2 uptake (O_2)	H_2 uptake (MeB)	H_2 evolution	H_2ase protein	Large subunit processing
Mutants in HoxK						
Cys62	Cys→Ser	+	+	-+	+	+
Cys64,65	Cys→Ser	+	+	+	+	+
Cys65	Cys→Ser	++	++	+	+	+
Cys232	His→Arg	-+	-+	-+	+	?
Cys235	Cys→Ser	+	+	+	+	+
Cys266	Cys→Ser	-+	-+	-+	+	?
Cys294	Cys→Ser	+	+	+	+	+
Cys297	Cys→Ser	+	+	+	+	+
Mutants in HoxG						
Cys431	Cys→Ser	++	++	+++	+	+
Cys434	Cys→Ser	-	-	?	+	+
His474*	His→Arg	-	-	?	+	?
His474*	His→Arg	-+	++	+	+	?
Cys937	Cys→Thr	-	-	-	+	?
	Cys→Leu	-	-	-	+	?
Mutants with modified genes						
HoxKG-	gene deletion	-	-	-	-	-
HoxZ-	gene deletion	++	++	++	+	+
HoxM-	gene deletion	-	-	-	+	?
*Eco*RI-KG	Restriction site inserted upstream KG	+++	+++	+++	+++	+++
pNif-KG	nif promoter inserted upstream KG	+++	+++	+++	+++	+++

+++ an activity similar to the wild-type; ++ less than the wild-type; + detected; -+ minor activity detected; - no activity; ? not corroborated; * amino acids possibly not serving as ligands to a metal cofactor. H_2 uptake was measured amperometrically in a Clark style electrode; H_2 evolution was detected using methyl viologen in a gas chromatography assay and amperometrically. Protein of HoxG was detected in an immunoblot using an antibody for *A. vinelandii* hydrogenase.