NOTICE

CERTAIN DATA CONTAINED IN THIS DOCUMENT MAY BE DIFFICULT TO READ IN MICROFICHE PRODUCTS.
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 $\text{H}_2$ generated by nitrogenase in biological $\text{N}_2$ fixation. This “recycling” of $\text{H}_2$ leads to an increased efficiency of $\text{N}_2$ fixation. Our research has focussed on investigations of the catalytic mechanism of the hydrogenases found in aerobic, $\text{N}_2$-fixing microorganisms such as Azotobacter vinelandii and the agronomically important Bradyrhizobium japonicum as well as microorganisms with similar hydrogenases. The hydrogenases isolated from these microorganisms are Ni- and Fe-containing heterodimers. Our work has focussed on three areas during the last grant period. In all cases, a central theme has been the role of inhibitors in the characteristics under investigation. In addition, a number of collaborative efforts have yielded interesting results.

**Investigations of Inhibitor Mechanisms**

In metalloenzymes such as hydrogenase, inhibitors often influence the activity of the enzyme through ligand interactions with redox centers, often metals, within the enzyme. Therefore, investigations of the ability of various compounds to inhibit an enzyme’s activity, as well as the mechanism of inhibition, can provide insight into the catalytic mechanism of the enzyme as well as the role of various redox centers in catalysis. We have investigated in detail four inhibitors of A. vinelandii and the results are summarized here. The influence of these inhibitors on the spectral properties of the enzyme are summarized in a later section.

**Acetylene.** In previous work, we determined that acetylene is a slow-binding, site-directed inhibitor of A. vinelandii and B. japonicum hydrogenase (Hyman and Arp, 1987). While still bound to the membranes, A. vinelandii hydrogenase can be obtained aerobically in an inactive, but catalytically competent, state. This aerobically prepared, inactive form of hydrogenase was not sensitive to acetylene, but could be made acetylene sensitive by reduction (leading to activation) of the enzyme. The results indicated that acetylene inhibition requires catalytically competent enzyme (Hyman et al., 1988). In continued studies of the mechanism of acetylene inhibition, we have investigated the ability of n-terminal alkynes to inhibit hydrogenase. Although inhibition was observed with propyne, the rate of inhibition was that expected from the level of acetylene which contaminated the propyne. Therefore, no substantial rate of inhibition could be associated with propyne. Likewise, n-butyne did not lead to any inhibition of hydrogenase activity. Ethylene was also not an inhibitor of hydrogenase. Thus, the inhibition was specific for acetylene. Our current model for the inhibitory effect of acetylene is that the binding of acetylene is associated with the loss of a proton from acetylene and the formation of a Ni acetylide. Reversal of the inhibition (which requires an overnight incubation) would therefore require abstraction of a proton from the medium and reformation of the C-H bond in acetylene. As a first test of this model, we have determined (by GC analysis and a chemical test) that acetylene is released from the enzyme during the recovery phase (e.g. no chemical transformation of the acetylene has occurred). Further tests of this model are described in the Experimental Plan section of the grant proposal.

Attempts to induce a covalent attachment of the acetylene at the active site (by treatment with bromine to initiate a radical formation in the acetylene) were unsuccessful. Acetylene is competitive with $\text{H}_2$ for binding to hydrogenase. Therefore, we asked if acetylene could also protect the enzyme from $\text{O}_2$ inactivation, as does $\text{H}_2$ (see below). When the enzyme was incubated in the presence of acetylene alone, then the acetylene was removed and the enzyme incubated in the presence of $\text{H}_2$, full activity was recovered. When the enzyme was incubated in the presence of acetylene, then $\text{O}_2$, then exposed to recovery conditions, full activity was again restored after several hours. Thus, acetylene, like $\text{H}_2$, can protect the enzyme from $\text{O}_2$ inactivation.

**Dioxide.** The effects of $\text{O}_2$ on hydrogenases are known to be complex. However, we have investigated these effects on the hydrogenase isolated from A. vinelandii and have placed...
many of the observations on a quantitative and rational basis. Basically, the effects of O2 on active hydrogenase can be separated into two categories. First, O2 is a rapid-equilibrium, reversible inhibitor of both the H2 oxidation activity and the exchange activity of the enzyme. The inhibition was noncompetitive versus the electron acceptor methylene blue and uncompetitive versus the substrate, H2. An inhibition constant of 5.5 μM was determined for the purified enzyme. The second effect of O2 is that of an irreversible inactivation of the H2 oxidation and exchange activities of A. vinelandii hydrogenase. This slower inactivation followed a first order process and gave a half-life of 5.9 minutes for purified enzyme. Surprisingly, the activity did not decay to zero; rather, a residual activity of about 10% of the original activity was obtained. Even after incubation of the enzyme in air for 24 hours, the activity remained at 10%. For membrane-associated enzyme, the half-life for inactivation was longer (46 min) and the residual activity was considerably higher (60%).

Various reagents were investigated for their ability to protect hydrogenase from inactivation by O2. The only reagent which could do so was H2. The protection by H2 was concentration dependent but saturated with as little as 0.2 kPa H2 in the gas phase. CO could not protect the enzyme from inactivation by O2 nor did it prevent H2 from protecting the enzyme. We propose that H2 and O2 are simultaneously bound to the enzyme in a "stalemate" such that O2 cannot carry out the reaction needed to inactivate the enzyme because of the presence of H2 and H2 cannot be activated either to allow exchange or the oxidation of H2 (e.g. with reduction of O2). This state of the enzyme (EH2, O2) has proven useful in subsequent investigations. (Seefeldt and Arp, 1989)

Aerobic purification of A. vinelandii hydrogenase. Most Ni and Fe containing hydrogenases are purified in an inactive state in the presence of air. Activation requires incubation under anaerobic conditions in the presence of a reductant. We have previously purified the hydrogenase from A. vinelandii in the absence of O2 under reducing conditions (2 mM dithionite). However, given the apparent convergence of many properties of the NiFe hydrogenases over the past few years, it was of interest to determine if the A. vinelandii hydrogenase could also be purified under aerobic conditions. We have worked out a purification protocol based on the anaerobic purification, but with slight modifications. The modifications are required because the inactive, but activatable, enzyme under air is not stable under conditions where the active enzyme under reducing conditions is stable. For example, the activatable form loses the ability to be activated when incubated at pH 5.0 overnight. Therefore, the overnight dialysis step was carried out at pH 7.4. With the modified protocol, we are able to purify to homogeneity an inactive form of the enzyme under air. We have examined a number of activators; H2 and dithionite are the most effective. The ability of the enzyme to be activated is not affected by acetylene, indicating that acetylene does not bind to this oxidized, inactive form of the enzyme. On the other hand, cyanide treatment leads to the loss of ability to activate the enzyme, indicating that cyanide can bind to this form of the enzyme. This is consistent with the mechanism of cyanide inactivation (see below). Once activated, the enzyme behaves essentially as the anaerobically purified enzyme with similar sensitivities to inhibitors and similar kinetic constants (Km's for H2 and electron acceptors).

The results indicate a similarity to other NiFe hydrogenases. Nonetheless, we do not intend to routinely purify enzyme under aerobic conditions. First and foremost, the yields are only 10% of those obtained in the anaerobic purification, even with the aforementioned modifications. Second, the enzyme preparations always contain mixtures of active and inactive enzyme. As a result, experiments are very dependent upon the history of the enzyme. Furthermore, the specific activities we obtain are not consistent and have not yet reached those obtained with the anaerobic purification. (Sun Jin-hua and Arp, Manuscript in preparation).

Cyanide. Cyanide serves as a strong ligand to most transition metals, and therefore has been used as an inhibitor of many metalloproteins. Nonetheless, there have been no extensive studies of the effects of cyanide on hydrogenases. In preliminary studies, it was apparent that cyanide has virtually no effect on active A. vinelandii hydrogenase. However, the characterization of an oxidized, inhibited state of the enzyme with H2 and O2 (EH2, O2) provided a new avenue for this investigation. When this form of the enzyme was incubated in the presence of KCN, an irreversible inacti-
The ability of the enzyme to be inactivated by NO was not reversible under non-turnover conditions. Rather, a time-dependent, irreversible inactivation occurred under non-turnover conditions. Again, this non-turnover inactivation took place at higher concentrations of NO. The inability of NO to influence the binding of O₂ suggests that NO and H₂ do not share the same binding site. This is similar to the results with O₂; NO is known to interact with O₂-binding proteins as an analog of O₂. Furthermore, the differences between turnover and non-turnover conditions suggest that the redox state of the clusters influences the effects of NO. (Hyman and Arp, 1990; submitted).

Electron Paramagnetic Resonance (EPR) Investigations of *A. vinelandii* Hydrogenase.

In collaboration with Gerrard Jensen and Philip Stephens at the University of Southern California, we have carried out an investigation of several states of *A. vinelandii* hydrogenase as influenced by various inhibitors and the substrate H₂. The spectrum of the enzyme "as isolated" (i.e. in the presence of dithionite) reveals a complex g=1.94 type of spectrum typical of a [4Fe-4S] cluster interacting with another paramagnetic (most likely, another [4Fe-4S] cluster in this case) (Fig. 3). This spectrum is like that of *B. japonicum* as isolated and similar to that of the particulate hydrogenase from *Alcaligenes eutrophus* when reduced. The spectrum is unlike that of *Desulfovibrio gigas* NiFe hydrogenase in the reduced state where no signals attributable to FeS centers are present at g<2. The basic features of this spectrum are unaltered by addition of CO, C₃H₄O₂ or H₂ to the sample, despite the fact that all three of these gases have been shown kinetically to interact with this form of the enzyme. Because all three of these compounds bind to the H₂ binding site of *A.
vinelandii hydrogenase, their lack of influence on the spectrum suggests that this signal does not arise from the site of H₂ interaction.

Oxidation of the enzyme with O₂ results in a rapid loss of the g=1.94 signal and formation of a new signal with a major feature centered at g=2.02 and satellite features at g=2.04 and 1.97 (Fig. 4). During a time course of O₂ inactivation, the major feature decreases in intensity, the satellite line at 2.06 disappears and the satellite line at 1.97 increases in intensity. Re-reduction of the sample with dithionite (which does not result in reactivation) results in reformation of the g=1.94 signal of the "as isolated" enzyme (Fig. 6). In addition, signals at 2.09, 2.20 and 2.22 are apparent and, by analogy to other hydrogenases, are assigned to Ni. Thus, O₂ inactivation does not alter the iron sulfur clusters(s) which give rise to the g=1.94 signal, but does appear to influence the Ni center. The g=2.02 feature of the oxidized enzyme is tentatively assigned to a [3Fe-4S] cluster. It appears that this center also retains the ability to undergo reduction following O₂ inactivation.

When we examined the EH₂,O₂ state of the enzyme, we found that the enzyme was in an oxidized state as indicated by the loss of the g=1.94 signal and the appearance of the g=2.02 signal (Fig. 5). However, the satellite lines were lost and the 2.02 signal was notably broadened. Thus, as suggested by the kinetic results, H₂ influenced the oxygenated state of the enzyme. When the enzyme was re-reduced, the EPR signal returned to that of the "as isolated" enzyme. When enzyme was incubated in the presence of C₂H₂ and O₂, the EPR spectrum was similar to that of EH₂,O₂. This further developed the concept that H₂ and C₂H₂ interact with the enzyme in similar manners.

Cyanide influenced the EPR spectrum of EH₂,O₂ primarily by causing a sharpening of the 2.02 signal. When reduced hydrogenase was treated with NO (100% for 5 min), the signal was altered to that typical of the Fe-S-NO complex described for other proteins. (Jensen, Seefeldt, Arp, Stephens, 1990, Manuscript in preparation)

UV-Vis Spectral Investigations of A. vinelandii Hydrogenase

Previous investigations of the UV-Vis spectra of hydrogenases have been limited to comparisons of "oxidized" and "reduced". We have undertaken an extensive investigation of the UV-Vis spectral properties of A. vinelandii hydrogenase. This study was facilitated by the well-characterized inhibitors made available by our studies. The reduced, "as isolated" enzyme reveals a spectrum typical of FeS proteins (Fig. 7). The spectrum exhibits a broad absorption envelope from the edge of the aromatic amino acid absorption to about 600 nm. This absorption increases in the EH₂,O₂ form of the enzyme (Fig. 8A). Difference spectra (EH₂,O₂ - Enzyme) revealed absorption maxima at 435 and 325 nm. Again, this is typical of oxidized-reduced spectra of iron sulfur clusters. The difference spectrum of aerobically-purified-hydrogenase minus activated aerobically-purified-hydrogenase revealed a similar spectrum.

When hydrogenase was oxidized (and inactivated) by treatment with O₂, the 435 nm peak was broadened considerably and a new peak was observed at 315 nm (Fig. 8B). Re-reduction of the enzyme resulted in decreases in the 435 and 325 nm peaks, but the 315 nm peak was not altered by treatment with reductant. The 315 nm peak, therefore, appears to be correlated with the irreversible inactivation by O₂.

When reduced enzyme was treated with H₂, the spectrum was identical to that of enzyme treated with dithionite (followed by removal of the dithionite under an Argon atmosphere) (Fig. 7). In contrast, treatment of reduced enzyme with acetylene resulted in a time-dependent appearance of a new absorption peak with a maximum at 492 nm (Fig. 9). This absorption was not likely to be due to oxidation of the FeS centers for two reasons. First, the absorption was nearly 60 nm shifted from that of the oxidized enzyme and of much less intensity. Second, when the acetylene-inhibited enzyme was treated with O₂, the absorptions typical of the oxidized FeS centers appeared. Furthermore, this peak did not appear when the enzyme was treated with acetylene in the presence of H₂ (which prevents the inhibition by C₂H₂). We are tentatively assigning this absorp-
Collaborative Projects

Inhibition of CO dehydrogenase from Rhodospirillum rubrum with NO and COS. Because of our interest in ligand interactions with metalloenzymes, we were interested to determine to what extent our observations with NiFe hydrogenase would extend to another NiFe enzyme, namely, CO dehydrogenase (CO-DH). In a collaborative effort with Scott Ensign and Paul Ludden (University of Wisconsin, Madison) we first examined the ability of acetylene to inhibit CO-DH. No inhibition was observed. We then examined the ability of NO to inhibit CO-DH. NO proved to be a potent inhibitor of CO-DH. It inactivates this enzyme in a time and concentration dependent manner. NO-treated apo-CO-DH (which lacks Ni) cannot be activated by addition of Ni, in contrast to the apoenzyme which has not been treated with NO. (Hyman, Ensign, Arp, Ludden; Manuscript in preparation).

We also investigated another potential inhibitor of this enzyme, namely, carbonyl sulfide (COS). COS proved to be a reversible inhibitor of this enzyme and gave a kinetic pattern versus CO which was indicative of competitive inhibition. COS inhibition was uncompetitive versus the electron acceptor, methyl viologen. COS does not appear to be an alternative substrate for CO-DH, given that the dye-oxidized enzyme is not reduced by COS. Rather, COS appears to be acting as a dead-end inhibitor. The significance of this is that it represents the first description of a rapid-equilibrium inhibitor of CO-DH which is competitive with CO. (Hyman et al., 1989). In contrast, COS does not inhibit A. vinelandii hydrogenase. CO-DH is not capable of catalyzing the isotope exchange reaction catalyzed by A. vinelandii hydrogenase. Thus, these two NiFe enzymes exhibit distinct catalytic properties and inhibition patterns.

Immunological comparison of Fe-only and NiFe Hydrogenases. In a collaborative project with Kornel Kovacs and Len Mortenson (Univ. Georgia), we investigated the immunological cross-reactivity of 11 purified hydrogenases with 7 polyclonal antibodies raised against specific hydrogenases or hydrogenase subunits. The comparisons were carried out using Western blots to provide good sensitivity and discrimination of the subunits of multimeric hydrogenases. The results revealed substantial immunological cross-reactivity between the various NiFe hydrogenases investigated, even though the representative hydrogenases included very different physiological groups. Somewhat surprisingly, we also observed limited cross-reactivity between antisera prepared against an Fe-only hydrogenase and 4 NiFe hydrogenases and between antiserum prepared against a NiFe hydrogenase and an Fe-only hydrogenase. Whether this reflects a common ancestry of portions of these enzymes or the similarity of H2 binding sites between these two classes of hydrogenases remains to be determined. (Kovacs et al., 1989)

Characterization of the structural genes of Azotobacter chroococcum. We determined the N-terminal amino acid sequences of the large and small subunits of A. vinelandii hydrogenase and B. japonicum hydrogenase. This information was used by Geoff Yates and coworkers (University of Sussex, Brighton, UK) to prepare an oligonucleotide for use in screening subclones of the genome which were known to contain HUP (hydrogen uptake) genes. In this way, the structural genes were identified and eventually sequenced. An analysis of the sequences was presented in the Literature Review section. (Yates et al, 1988; Ford et al., 1990)
The results of this DOE-sponsored project have contributed to our understanding of the catalytic mechanism of *A. vinelandii* hydrogenase. A group of inhibitors have been characterized. These provide information about the different types of redox clusters involved in catalysis and the roles of each. The results will also be applicable to other NiFe hydrogenases. One group has already used acetylene in a study of three desulfovibrio hydrogenases and shown that only the NiFe hydrogenases are inhibited. The inhibitor studies are also being extended to other enzymes, both in this laboratory and in others. We have characterized a number of spectral properties of *A. vinelandii* hydrogenase. The EPR signals associated with this hydrogenase in the reduced state are reminiscent of other NiFe dimeric hydrogenases such as *A. eutrophus*, but distinctly different from others such as *D. gigas* and *Chromatium vinosum*. Thus, while the NiFe dimeric hydrogenases are now recognized as a large group of similar enzymes, there are differences in the spectral and catalytic properties which are not explained by their similar redox inventories, identical subunit structures, immunological cross reactivity and conserved sequences. The inhibitors we have characterized are also proving of value in the spectral characterizations. Surprisingly, we only see a significant EPR signal attributable to Ni after the enzyme has been inactivated with O2 and then re-reduced (though not reactivated). No spectral perturbations (EPR or UV-Vis) of active enzyme can be attributed to binding of H2, even though H2 clearly binds to this form of the enzyme. Acetylene, which does not substantially perturb the EPR signal of active hydrogenase, does result in a new absorption envelope in the UV-Vis spectrum. Overall, the results of this project have revealed the complex interactions of the redox clusters in catalysis through studies of inhibitor mechanisms and spectral properties.

Manuscript, Meetings and Other Presentations of Research Results Resulting from This DOE-sponsored Project

1. Manuscripts

   A. *In Press or Submitted* at the time of the last renewal request (7/15/87).


   B. *Submitted and Published since last renewal request* (7/15/87)


Seefeldt, L.C. and D.J. Arp (1989) Oxygen effects on the nickel- and iron-containing hydrogenase from Azoto-

bacter vinelandii. Biochemistry 28, 1588-1596. (DOE supported)


II. Meetings

Research results from this DOE-sponsored project were presented at the following scientific conferences:

Hydrogenase Meeting, Unicoi, Georgia; September 1988 (DJA, LCS, MRH)

6th International C$_1$ Symposium; Gottingen, West Germany; August, 1989 (DJA)

UCLA Symposium: Inorganic Chemistry and Molecular Biology Interface; Taos, New Mexico; February 1990 (MRH)

8th Int. Symposium N$_2$ Fixation; Knoxville, Tennessee; May 1990 (DJA, SJH)

III. Other

The results of this research were presented in several seminars and shared informally with colleagues at several institutions. Materials (e.g. antibodies and purified enzyme) were also frequently shared with colleagues at several institutions.
**Figure 1**
NO (5 μM) Reversibly Inhibits Membrane-associated Hydrogenase H₂ Oxidation Activity

![Absorbance vs. Time Graph](image)

**Figure 2**
NO (250 μM) Irreversibly Inactivates Hydrogenase Activity

![Absorbance vs. Time Graph](image)

**Fig. 2.** EPR Spectra of NiFe Hydrogenases

![EPR Spectra Image](image)

**Fig. 3.** EPR Spectra of *A. vinelandii* Hydrogenase during O₂ Inactivation

![EPR Spectra Image](image)
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