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Hydrogenase isolated from *Azotobacter vinelandii* is an heterodimer (65 kDa and 35 kDa) and contains 12.6 Fe and 1.3 Ni per enzyme. The Fe is present in the form of [4Fe-4S] and [3Fe-4S] clusters and Ni is coordinated to the large subunit. To investigate the role of these centers by using inhibitor kinetics, a number of inhibitors of the *A. vinelandii* hydrogenase were further characterized in this dissertation, including acetylene (C_2H_2), oxygen (O_2), cyanide (CN^-), nitric oxide (NO), and Cu(II).

C_2H_2 remains bound to the large, 65 kDa subunit (and not to the small, 35 kDa subunit) of hydrogenase following denaturation as evidenced by SDS-PAGE and fluorography of $^{14}C_2H_2$-inhibited hydrogenase. C_2H_2 and radioactivity were released from hydrogenase during the recovery from inhibition. The release of C_2H_2 proceeds more rapidly than the recovery of activity. No transformation of C_2H_2 to another compound occurred as a result of the interaction with hydrogenase. The inhibition is remarkably specific for C_2H_2-- propyne, butyne and ethylene are not inhibitors.

Properties of inhibitors O_2, C_2H_2, CN^- and NO are characterized in terms of effects on the UV-vis spectra of the *A. vinelandii* hydrogenase. Inhibition of enzyme by O_2 results in the reversible appearance of ΔA_{435nm} peak and ΔA_{345nm} peak, but inactivation of enzyme by O_2 results in an irreversible appearance of ΔA_{315nm}, in

addition to the ΔA_{435nm} and ΔA_{345nm} peaks. The C_2H_2-inhibited enzyme showed ΔA peaks at 492, 338 and 289 nm that could be prevented by the presence of H_2. However, C_2H_2 inhibition did not affect the oxidation of the [Fe-S] clusters by O_2. CN^- inactivation caused increased absorption at 310 and 340 nm and decreased absorption at 380 nm. In CN^--inactivated enzyme, the oxidized [Fe-S] clusters could not be reduced by H_2, but the clusters were reduced by dithionite. Nitric oxide induced a broad absorption band in the 530-300 nm range and an increased absorption at 320 nm. Exposure to O_2 resulted in destruction of the [Fe-S] clusters.

A new inhibitor, Cu(II), of the *A. vinelandii* hydrogenase was characterized. Cu(II) (1 to 10 μM) irreversibly inactivated hydrogenase either under catalytic turnover conditions or when incubated in the absence of a substrate. Among H_2 oxidation, H_2 production and D_2/H^+ isotope exchange reactions, no major difference was observed in terms of sensitivity to Cu(II) inactivation. The Cu(II) inactivation required the presence of H_2 and a functional H_2 activation site. During the Cu(II) inactivation, the absorption of light by the [Fe-S] clusters was bleached while the absorption at 300 nm and 320 nm increased. The kinetic study indicated that the Cu(II) inactivation was a saturable process with a slow binding mechanism.

STRUCTURE AND FUNCTION OF HYDROGENASE FROM *AZOTOBACTER VINELANDII*

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CHAPTER I.

OVERVIEW

Introduction

Hydrogenases (hydrogen: acceptor oxidoreductase EC 1.18.99.1 & EC 1.12.2.1) comprise a group of enzymes that catalyze the oxidation or production of molecular hydrogen (H_2) according to the equation:

$$H_2 <===> 2H^+ + 2e^-$$

The oxidation of H_2 provides some organisms with supply of reductant which also may be used for energy generation by coupling to the reduction of different electron acceptors such as O_2, NO_3^{1-}, SO_4^{2-}, CO_2 and fumarate. Alternatively, H_2 production enables some organisms to dispose of excess reductant in the absence of electron acceptors other than protons. Hydrogenases have been found in a wide variety of prokaryotes including aerobes, facultative anaerobes, phototrophic organisms and strict anaerobes as well as in some eukaryotes such as algae, protozoan (Adams, et al., 1981) and higher plants (Torres, et al., 1986). Therefore, the hydrogenase is a central metabolic system for energy in many important prokaryotes and eukaryotes.

In the past years our concern with energy production has initiated a considerable amount of research into the hydrogenases. These studies have been extensively reviewed (Adams, 1990a, Adams, 1990b, Adams, et al., 1981, Bowien and Schlegel, 1981, Evans, et al., 1988, Fauque, et al., 1988, Hausinger, 1987, Houchins, 1984). To date, more than 40 hydrogenases have been purified from various microorganisms (Table I-1), and more

than 20 hydrogenases have been sequenced. A comparison of hydrogenase properties reveals a fairly heterogeneous group of enzymes which differ in molecular composition, specific activity in catalyzing H_2 production and H_2 oxidation, electron carrier specificity, cofactor content and sensitivity to inhibition or inactivation by O_2 or other inhibitors. However, they have one common feature, all are iron (Fe) containing proteins (with the exception of hydrogenase from methanogenic archaea which has no metals (Zirngibl, et al., 1992)), and a majority also contain nickel (Ni). Thus, the hydrogenases are divided into two major groups: (1) only Fe containing hydrogenases which are termed the Fe-only hydrogenases; (2) Ni and Fe containing hydrogenases which are termed the Ni-containing hydrogenase. In this chapter, a brief overview of structures and their functions of Ni-containing hydrogenases is presented. Hydrogenase was recognized almost 60 years ago as an enzyme capable of activating H_2, and so it has an illustrative history, reflecting in particular the development of our understanding of hydrogenase in general, and presenting questions for future studies. I therefore start this chapter with a historical perspective.

Historical Perspective

In 1839, de Saussure (de Saussure, 1839) first observed consumption of H_2 and O_2 in a soil sample. At the beginning of this century, the consumption or production of H_2 was confirmed to be due to the activities of bacteria. In 1906, Kaserer (Kaserer, 1906) isolated *Basillus pantotrophus*, and used pure bacteria to show the conditions for H_2 oxidation and H_2 production. By 1922, Ruhland (Ruhland, 1922) provided the first direct experimental evidence for the oxy-hydrogen reaction and showed that oxygen, not CO_2, was the electron acceptor for H_2. The consumption and production of H_2 was not regarded as a process catalyzed by enzymes until 1931, when Stephenson and Stickland (Stephenson and Stickland, 1931) found that *Escherichia coli* coupled the consumption

of H_2 to reduction of methylene blue (an artificial dye), and named the enzyme a hydrogenase.

Since 1931, methylene blue has been widely used in assays of hydrogenases. It is a useful tool for detecting hydrogenase, but its accurancy is affected by endogenous reductants. In 1934, Farkas et al. (Farkas, et al., 1934) showed that *E. coli* cells catalyzed an exchange reaction between H_2 and D_2O according to the reaction:

$$H_2+D_2O <==> HD +HDO$$

Deuterium exchange was later demonstrated to be useful for understanding the mechanism of hydrogen activation by hydrogenase. In 1954, based on the deuterium exchange results, Krasna and Rittenberg (Krasna and Rittenberg, 1954) proposed a heterolytic cleavage model for hydrogen activation. This model is still accepted now. However, deuterium exchange was not catalyzed by oxidized enzyme. In 1955, Tamiya et al. (Tamiya, et al., 1955) developed a procedure for the manometric determination of hydrogenase-catalyzed H_2 evolution using methyl viologen as the electron carrier and sodium dithionite as the electron donor. This new assay eliminated any inhibitory effects of O_2, which were removed by the powerful reducing mixture of dithionite and the viologen, and it enabled more extensive and quantitative estimations of hydrogenase activity in a wide range of bacteria.

In 1943, Hoberman and Rittenberg (Hoberman and Rittenberg, 1943) found that hydrogenase activity of *Proteus vulagaris* cell suspensions was inhibited by oxygen, cyanide and CO. The CO effect was reversed by light. In 1944, Waring and Werkman (Waring and Werkman, 1944) showed that *Azotobacter* lack hydrogenase activity when grown on Fe-deficient media. From the 1940's onwards, a lively controversy existed as to the nature of hydrogenase and its prosthetic group. In 1963, Sadana and Rittenberg (Sadana and Rittenberg, 1963) were the first to propose that the active center of hydrogenase might consist of a ferrous-sulfhydryl complex. In the same year, the

presence of iron and acid-labile sulfide was demonstrated in bacterial ferredoxin (Buchanan, et al., 1963, Lovenberg, et al., 1963) which was discovered by Mortenson et al in 1962 (Mortenson, et al., 1962). Curiously, the presence of ferrous-sulfhydryl complex in the hydrogenase was not realized until seven years later. Not until 1971, when Nakos and Mortenson (Nakos and Mortenson, 1971a, Nakos and Mortenson, 1971b) purified hydrogenase from *C. pasteurianum* and showed it contained 4Fe and 4 acid labile sulfide (S^{-2}) and exhibited EPR signals characteristic of reduced ferredoxins, was the presence of [Fe-S] clusters unequivocally demonstrated.

A limiting factor for demonstrating the presence of a ferrous-sulfhydryl complex was the lack of purified hydrogenase. In 1950, Joklik (Joklik, 1950) made the first attempt to purify hydrogenase from *E. coli* , but he obtained only partially purified hydrogenase. One of the major problems in the purification field up to the mid-50's was that the organisms under study all contained membrane-bound hydrogenases, which made extensive purification difficult to achieve. A major breakthrough was the discovery that soluble preparations of hydrogenase could be obtained from strict anaerobes such as *Clostridium* and *Desulfovibrio* sp (Peck and Gest, 1957, Sadana and Jagannathan, 1956, Shug, et al., 1954). With an improvement in purification methods and the use of strictly anaerobic techniques, the first reasonably pure preparations of hydrogenase were obtained from *C. pasteurianum* in 1971(Nakos and Mortenson, 1971a, Nakos and Mortenson, 1971b).

In 1965, Bartha and Ordal (Bartha and Ordal, 1965) discovered that nickel was essential for chemoautotrophic growth of hydrogen-oxidizing bacteria. In 1980, Lancaster (Lancaster, 1980) observed novel EPR signals from the membranes of methanogenic bacteria, and tentatively assigned them to a Ni(III) species. By 1982, this EPR signal was confirmed to arise from the Ni atom by the following observations: (1) ^{63}Ni inserts into membrane bound and soluble hydrogenases from *A. eutrophus* hydrogenase (Friedrich, et

al., 1982); (2) Hydrogenase from *Methanobacterium thermoautotrophicum* contains 1 Ni atom per enzyme (Graf and Thauer, 1981); (3) ^{61}Ni (I=3/2) gives rise to hyperfine structure of the novel EPR signals in the ^{61}Ni-substituted hydrogenase from *M. bryantii* (Lancaster, 1982), from *M. thermoautotrophicum* (Albracht, et al., 1982b), and from *D. gigas* (Cammack, et al., 1982, LeGall, et al., 1982). Therefore, in addition to iron-sulfur clusters, Ni is an essential component of some hydrogenases. This discovery dramatically changed our understanding of hydrogenase and it also sparked a flurry of interest in determining any additional metal content of hydrogenase. Selenium was first found in hydrogenase from *Methanococcus vannielii* in the form of selenocysteine (Yamazaki, 1982). To date, Se, Cu and Zn have been found in a limited number of hydrogenases, although their roles are completely unknown (Hausinger, 1987).

In the 1980's, another development was the cloning and sequencing of hydrogenase genes. In 1985, Voordouw and Brenner (Voordouw and Brenner, 1985) first cloned and sequenced the gene coding for *D. vulgaris* hydrogenase (an Fe-only hydrogenase). At that time, this work confirmed the presence of heterodimeric Fe-hydrogenase. In 1987, two genes coding for nickel-containing hydrogenases from *D. gigas* and *D. baculatus* were sequenced (Li, et al., 1987, Menon, et al., 1987). To date, hydrogenase structural genes have been sequenced from more than 20 microorganisms. The deduced amino acid sequences are useful for studying the catalytic mechanism of hydrogenase. Alignment of these sequences has revealed some interesting conserved regions (see III-E sections), which become the target of site-directed mutation for studying the structure and function of hydrogenase.

Another power exhibited by sequencing hydrogenase genes is to discover components of the hydrogenase operon. In 1988, Sayavedra-Soto (Sayavedra-Soto, et al., 1988) sequenced a 3.4kb DNA fragment which included the genes encoding the small and large subunit in *B. japonicum*. In that sequence, the gene for the large subunit is

immediately followed by a ribosome-binding site and an ATG initiator codon. But the additional open reading frames were not realized until 1990 when Menon NK et al. (Menon, et al., 1990) cloned a 6.0 kb DNA fragment that contained the entire *hya* operon. They found that four additional open reading frames (ORFs) sit immediately downstream of the large subunit gene. Within two years, several hydrogenase operons were shown to contain additional ORFs (Przybyla, et al., 1992). The characterization of these ORF products could influence our understanding of hydrogenase in many respects: catalytically active unit, physiologically active unit, and hydrogenase formation.

Structure of Ni-Containing Hydrogenases

The Nickel-containing hydrogenases have now been isolated and characterized to varying degrees from well over a dozen different organisms, including photosynthetic bacteria, methanogens, sulfate reducing bacteria, colon bacteria, aerobic hydrogen bacteria, aerobic N_2-fixing bacteria and extremely thermophilic archaebacteria (Table I-1). The results reveal a remarkable diversity in subunit composition and prosthetic groups.

Subunit Composition

Among the Ni-containing hydrogenases, a few of them are monomers or multimers, whereas majority of them are heterodimers, with a large subunit of about 60,000 daltons and a small subunit of about 30,000 daltons. This diversity is shown in Table I-1.

Table I. 1. Microbial Hydrogenases

Microorganism	Form[a]	Subunits[b]	Metal/enzyme	Specific Act. (ev/up)[c]	Ref.
Fe only					
Clostridium pasteurianum					
(I)	S	*64	20-22 Fe	5500/24000	(a, b)
(II)	S	55	14Fe	10/34000	(a, b)
Megasphaera elsdenii	S	58	16Fe	7000/9000	(b, c)
Desulfovibrio vulgaris Hildenborough	S	*46, 10	9-15Fe	10400/50000	(b, d, e)
NiFe					
Desulfovibrio gigas	P	*62, 26.	12Fe/1Ni	420/1200	(f, g)
Desulfovibrio vulgaris (2)	M	85, 45	4Fe/0.3Ni	112/328	(h)
Miyazaki	M	*59, 28	8Fe/--	--/--	(i)
Desulfovibrio desulfuricans Norway	M	60, 27	6Fe/Ni	70/200	(j, k)
ATCC	S	78	8Fe/0.6Ni	--/97	
		76	11Fe/0.6Ni	--/152	(l)
NRC49001	S	52	12Fe/NR	900/NR	(k)
Desulfovibrio multispirans	S	58, 25	11Fe/0.9Ni	790/586	(n)
Desulfovibrio africanus	S	65, 27	12Fe/0.9Ni	570/2800	(o)
Chromatium vinosum	S	69	4Fe/0.5Ni	42.5/425	(p, q)
Rhodobacter capsulata	M	*67, 31	4Fe/0.2Ni	1.3/30	(r, s, t)
Rhodospirillum rubrum	M	65	4Fe/--	33/NR	(t, ccc)
Thiocapsa reseopersicina	M	64, 34	4Fe/1Ni	60/46	(t, u)
Methanobacterium thermoautotrophicum					
F_{420}	S	*47, 31, 26	14Fe/0.6Ni 0.8FAD	--/49	(v, w)
non-F_{420}	S	*57,45,42,33	Fe/Ni	ND	(x, y)
(α,β)		* 52,40	Fe/1Ni	--/180	(y, z)

Table I-1: Microbial Hydrogenases (Cont.)

Microorganism	Form[a]	Subunits[b]	Metal/enzyme	specific Act. (ev/up)[c]	Ref.
Methanobacterium formicicum					
F_{420}	S	43,34,24	20Fe/3Ni	1.2/49	(aa)
non-F_{420}	S	48,38	10Fe/1Ni 1Zn/1Cu	50.1/170	(aa, bb)
Methanosarcina barkeri	M	60	9Fe/0.7Ni 1FMN	270/--	(cc)
Alcaligenes latus	M	67,34	2Fe/0.5Ni	0.11/113	(dd)
Alcaligenes eutrophus					
MB	M	*61,30	8Fe/0.7Ni	--/170	(ee, ff)
Soulable	S	*67,55,26,23	16Fe/2Ni 1FMN	--/57	(ff, gg)
Mutant HF14	S	*57	2-3Fe/0.2-1.4Ni	NR/29	(hh)
Nocardia opaca	S	64,56,31,27	14Fe/4Ni 1FMN	--/45	(ii)
Azotobacter vinelandii	M	*67,31	12.6Fe/1.3Ni	4.97/233	(jj, m)
Azotobacter chroococcum	M	*67,34	4.4Fe/Ni	--/6	(kk, ll)
Bradyrhizobium japonicum	M	*64,35	6.5Fe/0.6Ni	--/65	(mm, nn, oo)
Escherichia coli					
(I)	M	*64,35	12Fe/0.6Ni	1.6/11	(i, pp)
(II)	M	*61,35	12Fe/3Ni	20/630	(i, pp)
(α_2)	M	*65x2	2-3Fe/--	35/49	(qq)
Vibrio succinogenes	M	60,30	11-20Fe/1Ni	NR/660	(rr)
Thermogoga maritima	S	67.6x4	20Fe/--	56/78	(ss)
Pyrococcus furiosus	S	46,27,24	31Fe/1Ni	2900/261	(tt)
NiFeSe					
Methanococcus vannielii	S	42	2Ni/3.8Se	ND	(uu)

Table I-1: Microbial Hydrogenases (Cont.)

Microorganism	Form[a]	Subunits[b]	Metal/enzyme	Specific Act. (ev/up)[c]	Ref.
Methanococcus voltae	S	55,45,37,27	4.5Fe/0.7Ni/0.7Se	--/10	(aaa)
Desulfovibrio baculatus	P	*58,61	10Fe/0.7Ni/0.7Se	18/--	(vv, ww, xx)
	M	62,27	10Fe/0.9Ni/0.9Se	28/--	(xx)
	S	54,27	8Fe/0.5Ni/0.6Se	430/120	(xx, yy)
ATCC9974	S	100	1Ni/1Se	527/NR	(zz)
Desulfovibrio salexigens	P	62,36	15Fe/1Ni/1Se	60/NR	(bbb)
Desulfovibrio vulgaris					
(1)	M	*85,45	4Fe/0.3Ni/0.3Se	3.9/5.3	(h)
(3)	M	86,45	4Fe/0.3Ni/0.3Se	1850/560	(h)
Desulfovibrio desulfuricans Norway	S	56,29	10Fe/0.8Ni/0.6Se	705/--	(k)

ND, not determined; NR, no record. [a] S=soluble, P=periplasmic and M=membrane-bound. [b] Subunits molecular masses in kilodaltons. [c] Specific activities are the highest reported values. ev=μmoles H_2 evolved·min^{-1}·mg^{-1}; up=μmoles H_2oxidized· min^{-1}·mg^{-1}. * This subunit has been sequenced in its gene. (a) (Adams, 1990b); (b)(Adams and Mortenson, 1984); (c) (Mayhew, et al., 1978); (d) (Voordouw and Brenner, 1985); (e) (Van der Westen, et al., 1978); (f) (Hatchikian, et al., 1978); (g) (LeGall, et al., 1982); (h) (Lissolo, et al., 1986); (i) (Sawers and Boxer, 1986); (j) (Lalla-Maharajh, et al., 1983); (k) (Rieder, et al., 1984); (l) (Kruger, et al., 1982); (m) (Sun, et al., 1992); (n) (Czchowski, et al., 1984); (o) (Niviere, et al., 1986); (p)(Albracht, et al., 1982a); (q)(Van Heerikhuizen, et al., 1981); (r) (Seefeldt, et al., 1987); (s)(Colbeau and Vignais, 1983); (t) (Gogotov, 1986) (u) (Tigyi, et al., 1986); (v) (Livingston, et al., 1987); (w) (Fox, et al., 1987); (x) (Walsh and Orme-Johnson, 1987); (y) (Kojima, et al., 1983); (z) (Reeve and Beckler, 1990); (aa) (Jin, et al., 1983); (bb) (Adams, et al., 1986); (cc) (Fauque, et al., 1984); (dd) (Pinkwart, et al., 1983) (ee) (Schink and Schlegel, 1979); (ff) (Friedrich, et al., 1982); (gg) (Schneider and Cammack, 1978); (hh) (Hornhardt, et al., 1986); (ii) (Schneider, et al., 1984); (jj) (Seefeldt and Arp, 1986); (kk) (Yates, et al., 1988); (ll) (van der Werf and Yates, 1978); (mm) (Arp, 1985); (nn) (Harker, et al., 1984); (oo) (Stults, et al., 1984); (pp) (Ballantine and Boxer, 1986); (qq)(Francis, et al., 1990); (rr) (Unden, et al., 1982); (ss) (Juszczak, et al., 1991); (tt) (Adams, 1990a); (uu)(Yamazaki, 1982); (vv) (Menon, et al., 1987); (ww) (Menon, et al., 1988); (xx) (Teixeira, et al., 1987); (yy)(Lespinat, et al., 1986); (zz) (Teixeira, et al., 1985b); (aaa) (Muth, et al., 1987); (ccc) (Adams and Hall, 1979).

However, there is no clear definition of what constitutes a subunit of a hydrogenase. "Subunit" could refer only to those polypeptides that are necessary for the activation of H_2, or could be any protein that is directly associated (i.e. co-purifies) with hydrogenase activity and is approximately stoichiometric. For many hydrogenases, we do not know what is their natural electron acceptor or donor. Therefore, no ideal system exists for examining how many and what kinds of peptides are necessary for H_2 oxidation. Also, it is possible that a protein may not play any role in hydrogenase activity *in vivo*, although it is associated with hydrogenase. In the literature, the definition of subunit is based on the co-purification. This could be misleading because purification could miss polypeptides which are components of the enzyme. In previous purifications, the hydrogenases from *A. vinelandii* (Kow and Burris, 1984), and *B. japonicum* (Arp and Burris, 1979) were isolated as monomers. Later on, with improvement of purification and application of protease inhibitors, these enzyme were purified in the dimer forms (Arp, 1985, Harker, et al., 1984). The dimers have a 10-fold increase in specific activity compared to the monomer. The hydrogenase from photosynthetic bacterium, *R. rubrum* was reported to be monomer. But recently, reinvestigation show that it is a dimer (Koch, et al., 1992). Analyses of hydrogenase genes system has shown several ORFs located downstream of the ORFs that encode the large subunit and small subunit. The mutation of some ORFs affected H_2 oxidation *in vivo* (Maier, et al., 1993, Menon, et al., 1991, Sayavedrasoto and Arp, 1992). The dimeric hydrogenases could have a more complex subunit composition *in vivo*. The assignment of subunit compositions is in a state of flux.

Metal Content and other Prosthetic Groups

In addition to the Ni and Fe, some NiFe hydrogenases contain selenium, and some contain FMN or FAD (Table I-1). Thus, the NiFe hydrogenases can be divided into three subgroups: NiFe hydrogenase; NiFeSe hydrogenase; and NiFe-Flavin hydrogenases.

Iron Content The content of iron in the Ni-containing hydrogenase is extremely variable. General colorimetric method of protein determination overestimated the amount of protein in the enzyme sample leading to an underestimate of the content of iron. In addition, accurate iron determinations are limited by the lack of accurate molecular weights, and of pure enzyme. Also, apoprotein could be present in purified preparations, i.e. protein lacking a full complement of Fe. These underestimated numbers could be reflected by the number of Ni (less than 1) in the Ni-containing hydrogenases. If we look at the ratio of iron to nickel, we find the majority of Ni-containing hydrogenases could contain more than 11 atoms of iron per enzyme. This number may account for at least two [4Fe-4S] and one [3Fe-4S] cluster present in the Ni-containing hydrogenases. In fact, the number and type of Fe-S clusters is variable in the Ni-containing hydrogenases, with [4Fe-4S] type clusters common, and some examples of [2Fe-2S] and [3Fe-4S] clusters.

[2Fe-2S] Cluster The [2Fe-2S] cluster has been reported to be present in hydrogenases with more than two subunits, such as soluble NAD-reducing hydrogenase in *N. opaca* (Schneider, et al., 1984) or in *A. eutrophus* (Friedrich, et al., 1982), and soluble F_{420} reducing hydrogenase from *M. thermoautotrophicum*. (Fox, et al., 1987, Livingston, et al., 1987). These enzymes, in the reduced state, gave rise to two types of EPR spectra: (1) a signal typical of a ferredoxin type [2Fe-2S] cluster was present at more than 40K; and (2) a complex spectrum at 13K, which had additional featues at g=2.004, 1.93, and 1.86. Quantitation of the signals indicated one [2Fe-2S] cluster present in these tetrameric enzymes from *N. opaca* and *A. eutrophus* (Schneider, et al., 1984). Redox titration indicated that the [2Fe-2S] had a high redox potential (-285 mV), which is much higher than that of the [4Fe-4S] cluster in these enzymes (E_m for [4Fe-4S]: -420 mV (*N. opaca*) or -445 mV (*A. eutrophus*)). Because of the higher redox potential and location in the large dimer (see below), the [2Fe-2S] cluster has been proposed to react as an electron carrier between the [4Fe-4S] cluster and flavin.

[3Fe-4S] Cluster Although no extensive studies show how many Ni-containing hydrogenases contain the [3Fe-4S] cluster, it has been shown that the best characterized hydrogenases from *D. gigas* (Huynh, et al., 1987, Teixeira, et al., 1989), *A. vinelandii* (Seefeldt, 1989), *A. eutrophus* (membrane bound,(Schneider, et al., 1983)), *C. vinosum* (Albracht, et al., 1983), *D. desulfuricans* (Kruger, et al., 1982, Lalla-Maharajh, et al., 1983) contain a [3Fe-4S] cluster. In some cases, the presence of the [3Fe-4S] could arise from the aerobic degradation of a [4Fe-4S] clusters (Adams, et al., 1986). An inter-conversion between the [3Fe-4S] and [4Fe-4S] cluster was once regarded as a model for regulating the hydrogenase activity in membrane-bound hydrogenase from *A. eutrophus* (Schneider, et al., 1983), similar to aconitase. The [4Fe-4S] cluster was proposed to represent the active form of enzyme and the [3Fe-4S] to represent the inactive form. This model does not seem applicable to all the Ni-containing hydrogenases. Evidence from Mossbauer and EPR studies indicated that the soluble NiFeSe dimeric hydrogenase from *D. baculatus* did not contain a [3Fe-4S] cluster (Bell, et al., 1984, Teixeira, et al., 1990).

The [3Fe-4S] has been extensively studied in *D. gigas* hydrogenase. The following discussion is based on studies of *D. gigas* hydrogenase (Teixeira, et al., 1985a, Teixeira, et al., 1989). In the EPR spectra, the [3Fe-4S] cluster gives rise to an isotropic signal with a g values of 2.02 in the oxidized form. During reduction, this signal disappears and a new broad signal appears in the low field region (g=12). Appearance of g=12 signal follows a Nernst profile with E'_0=70±10 mV and n=1 values that are identical to those reported for the reduction of the [3Fe-4S] culster. Therefore, like the [3Fe-4S] cluster in other proteins, the [3Fe-4S] cluster in *D. gigas* hydrogenase existed both in the oxidized (1+) and reduced (0) states. Midpoint redox potential of the $[3Fe\text{-}4S]^0/[3Fe\text{-}4S]^{+1}$ couple has been determined to be -70 mV and pH independent. During further reduction, the g=12 signal disappeared and another broad EPR signal termed Fe-S signal B at even lower magnetic field starts developing. Almost in parallel

with the development of the Fe-S signal B, the Fe-S signal B' that has been assigned to the reduced [4Fe-4S] clusters developed. The Mossbauer date indicated that the [3Fe-4S] cluster remain at the same oxidation state during further reduction. The disappearance of the g=12 signal can not be a result of further reduction of the [3Fe-4S] cluster. It is suggested that the [3Fe-4S] cluster is sensitive to the redox state of other centers in the enzyme, either through a direct interaction or through conformation changes of the protein, and these changes may cause a small modification of the cluster's electronic structure resulting in the observed shift of the resonance.

In the Mossbauer spectra, the reduced [3Fe-4S] cluster exhibited two sharp quadrupole doublets in the absence of a magnetic field. The intensity ratio of the two doublets is 2 to 1 with more intense doublet having larger ΔE_Q and δ. The parameters from the Mossbauer spectra indicated that the reduced [3Fe-4S] cluster consists of a high spin ferric ion (doublet II) and a pair of iron ions with formal oxidation state of +2.5 (doublet I) i.e. the electron is shared by two ferric ions as reported for the [3Fe-4S] clusters found in other proteins. However, the ligand environment of the [3Fe-4S] cluster in *D. gigas* hydrogenase may be different from those in other proteins, because the isomer shift of doublet II, 0.39 mm/s, is significantly larger than those, 0.26-0.30 mm/s, observed for other [3Fe-4S] clusters. Also, the [3Fe-4S] cluster is magnetically isolated from the Ni paramagnetic center, but it interacts with the reduced [4Fe-4S] clusters.

[4Fe-4S] Cluster It appears that all Ni-containing hydrogenases contain at least two [4Fe-4S] clusters which are of the ferredoxin type. The hydrogenases from the photosynthetic bacteria, *C. vinosum* and *Thiocapsa roseopersicina*, were originally proposed to contain just a single [4Fe-4S] cluster (Van der Zwaan, et al., 1985, Zorin, 1986). But more recent analyses have shown that these enzymes contain at least two [4Fe-4S] clusters per Ni atom (Cammack, et al., 1989, Van derZwaan, et al., 1987).

The nature of the [4Fe-4S] clusters in the Ni-hydrogenases is not known, since, in contrast to the Ni center, they are not detectable by EPR spectroscopy in most redox states of these enzymes. The Mossbauer spectra of the two [4Fe-4S] clusters of *D. gigas* and *D. baculatus* hydrogenases in their oxidized form (+2 states) are similar to those of other $[4Fe\text{-}4S]^{+2}$ clusters. In the reduced state, the [4Fe-4S] cluster is present as superposition of two subspectral components (site 1 and site 2). Each component consists of two iron atoms. Two components are antiferromagnetically coupled (Teixeira, et al., 1990, Teixeira, et al., 1989), quite similar to bacterial ferredoxin type cluster. However, the hyperfine-coupling constants for the site 1 in each center are unusually small (Teixeira, et al., 1990, Teixeira, et al., 1989). This could be suspected to relate to S=3/2 state of [4Fe-4S] clusters. Studies from protein and model compounds showed that the $[4Fe\text{-}4S]^{1+}$ clusters can exist in many spin states other than the usual spin 1/2 state. Small hyperfine coupling constants were detected for the S=3/2 state (Carney, et al., 1988a, Carney, et al., 1988b, Lindahl, et al., 1985, Lindahl, et al., 1987). In previous EPR studies, a broad signal (termed Fe-S signal B') in the low field was observed to correlate with the reduction of the two [4Fe-4S] clusters (Cammack, et al., 1987, Fernandez, et al., 1986). But the Mossbauer spectra of the reduced [4Fe-4S] clusters are quite different from those of a S=3/2 $[4Fe\text{-}4S]^{1+}$ cluster (Teixeira, et al., 1990). The primary structure of these hydrogenases showed that the arrangements of the cysteine residues in these enzyme are rather different from the arrangements found in bacterial ferredoxins. Therefore, the most plausible explanation for the unusually small hyperfine-coupling constants is that hydrogenases have altered structural features around the [4Fe-4S] clusters (Teixeira, et al., 1990).

It is not, perhaps, surprising that the structure around the two [4Fe-4S] clusters is so variable between Ni-containing hydrogenases, if the EPR signals that were assigned to the [4Fe-4S] clusters are considered together. In contrast to *D. baculatus* hydrogenases and *A. vinelandii* hydrogenase, the *D. gigas* hydrogenase did not exhibit the typical

"g=1.94" type EPR signal of a $[4Fe\text{-}4S]^{+1}$ cluster (Seefeldt, 1989, Teixeira, et al., 1990, Teixeira, et al., 1989). The membrane-bound hydrogenase from *A. eutrophus* showed a complex EPR signals with average values greater than 2 when in the oxidized form. This complicated spectra has been assigned to the interaction between the Ni center and the [4Fe-4S] cluster (Albracht, et al., 1984, Cammack, et al., 1986, Schneider, et al., 1983). When the hydrogenase was fully reduced, it gave rise to a spectrum with principal features at g=1.86, 1.92 and 1.98, and 2.02 with additional broad lines to high and low field. This spectrum was explained as a triplet spectrum arising from a spin-spin interaction between two paramagnetic [Fe-S] clusters (Schneider, et al., 1983). The *A. vinelandii* hydrogenase has a similar EPR property to the *A. eutrophus* hydrogenase (membrane bound form) (Seefeldt, 1989). In the Mossbauer spectra, the reduced *D. gigas* hydrogenase exhibits two distinct reduced [4Fe-4S] clusters with different E_m values: -290 mV and -330 mV at pH 7.0. The one with higher redox potential was labeled Fe-S center I and the other, Fe-S center II. For the hydrogenase from *D. baculatus*, the Mossbauer spectra showed that its hydrogen-reduced form still contain an oxidized [4Fe-4S] center which yielded a diamagnetic component (Teixeira, et al., 1990).

Ni Center It has been noted that the NiFe hydrogenases from *Rhodobacter capsulata, A. vinelandii, B. japonicum, A. eutrophus* and *E. coli.* have low Ni contents (less than 1 atom /molecule). Re-examination of protein concentration in the hydrogenase from *A. vinelandii* by using quantitative amino acid analysis revealed that the Lowery methods of protein determination overestimated the amount of protein by a factor of 1.91(±0.32) (Sun et al. 1992). Correcting for this error, and using the molecular weight determined by physical methods and amino acid analysis (106,000), *A. vinelandii* hydrogenase contain 1.30 (±0.22) mol Ni/mol hydrogenase. The original preparations of this enzyme had specific activities in the H_2 oxidation assay about 124 μmol of H_2 /min·mg protein, and contained about 0.68 mol Ni/mol hydrogenase (Seefeldt and Arp, 1986).

Similarly, the hydrogenases from a *R. capsulata*, *A. eutrophus*, *B. japonicum*, and *E. coli* may also contain more than 1 atom per enzyme.

Selenium Eight hydrogenases isolated from 5 different microorganisms contain selenium. The selenium appears to be approximately stoichiometric with the nickel in these hydrogenases. The studies from NiFeSe hydrogenase of *Methanococcus vannielii* and *Desulfovibrio baculatus* (Menon, et al., 1988, Yamazaki, 1982) indicated that selenium might be present as selenocysteine. The selenocysteine is coded for on the DNA by the normal stop codon TGA. Thus, the selenocysteine is incorporated during translation (Chambers, et al., 1986, Zinoni, et al., 1986).

The role of selenium in hydrogenases is unknown. In glutathione peroxidase, the selenium may undergo redox changes upon substrate addition, suggesting a catalytic role (Wendel, et al., 1975). But in the case of hydrogenase case such a role seems unlikely. Boursier et al (Boursier, et al., 1988) observed a two-fold stimulation of hydrogenase activity when selenium was added to *B. japonicum* cells grown chemolithoautrophically related to cells grown in selenium free medium. However, no TGA codon (which codes for selenocystein) was found in the structural genes for this enzyme (Sayavedra-Soto, et al., 1988).

Others Other prosthetic groups that have been identified in hydrogenases include FMN and FAD. Most of the flavin containing hydrogenases are multimeric enzymes (with exception of hydrogenase from *Methanosarcina barkeri*). The flavin containing hydrogenases can reduce NAD^+, suggesting a role for the flavin in diaphorase activity. Stoichiometric quantities of copper (Cu) and Zinc (Zn) have also been identified in the non-F_{420} reducing hydrogenase isolated from *Methanobacterium formicium*, although these metals have no known functions in this enzyme (Adams, et al., 1986, Jin, et al., 1983).

Structure and Function of the Ni Center

The structure of the Ni center is unknown. The EXAFS and EPR studies performed in hydrogenases from *C. vinosum*, *D. gigas* and *M. thermoautotrophicum* (Cammack, et al., 1988, Lindahl, et al., 1984, Scott, et al., 1984) have suggested that Ni is 5- to 6-coordinate and bound by at least one and more likely three or four sulfur atoms, but not to Fe. The ESEEM studies of three hydrogenases (Cammack, et al., 1989, Chapman, et al., 1988, Tan, et al., 1984) have all indicated a weak interaction between the Ni center and a ^{14}N nucleus. In one case this was assigned to one N atom of a bound flavin (Tan, et al., 1984)(Tan et al 1984), and in the other two to the distal N of a histidine imidazole (Cammack, et al., 1989, Chapman, et al., 1988). Considering the changes of Ni EPR signals during activation and catalysis of enzyme (from Ni signal A to B and C, see (Teixeira, et al., 1989)), conserved regions in primary structure of enzyme, and effects of site directed mutagenesis on the activity of hydrogenase I from *E. coli* (Przybyla, et al., 1992), another structural model has been proposed by Przybyla et al (1992). In this model, the Ni center in isolated enzyme (inactive form), which gives rise to the Ni signal A, has a pseudooctahedral geometry with six ligands: one nitrogen ligand from Arg, one oxygen ligand form Asp, and four thiolate ligands from Cys. Two of the thiolate ligands are equatorial liganding, and another two are axial liganding. During activation by incubation with H_2, one of the axial thiolate ligands is replaced by OH^- resulting in the structure responsible for the Ni signal B species. This structure then reacts with H_2 to yield an EPR silent species. In this structure, the OH^- ligand is replaced by H_2, H_2 is activated and the loss of an electron and proton result in a structure responsible for Ni signal C. The axial ligands are sulfur and hydride. The equatorial ligands are the same as the Ni signal A species.

The Przybyla model gives a good explanation about the process of hydrogenase activation and H_2 activation. However, several questions remain unresolved.

Assignment of Redox State of Ni There is controversy as to whether the nickel cycles between four (III to 0), three (III to I) or two (III, II) redox states. The Ni species responsible for Ni signal A and Ni signal B are assigned to Ni(III), with the odd electron in the d_z2 orbital. However, Kumar et al (Kumar, et al., 1989) proposed that the Ni signal A arises from an Ni(II) center (S=1) that is spin coupled to a thiol radical to yield a S=1/2 system on the basis of the properties of novel Ni thiolate complexes. Upon reduction by H_2, both signals disappear and a Ni-signal C is observed. The g values of the Ni-signal C ($g_x \neq g_y > g_z \approx 2$) suggest that its unpaired electron also is associated with the d_z2 orbital of the nickel. However, whether the oxidation state of Ni-C is Ni(I) or Ni(III) is still in debate. Recently, Huang et al (Huang, et al., 1993) found that Ni(II)-substituted *P. furiosus* rubredoxin (Ni(II)-Rd) exhibited both the Ni-C and Ni-C* EPR type signals which were observed in *D. gigas* hydrogenase. The results support a Ni(III) assignment for both Ni-C and Ni-C* EPR signals and suggest that the Ni-C signal corresponds to a Ni(III) center with square pyramidal or tetragonally elongated octahedral coordination involving four cysteinyl-S ligands and one equatorially-bound H^-.

Site for Proton Exchange Kinetic studies suggest that H_2 activation by hydrogenase involves heterolytic cleavage of H_2, with the possible formation of a metal hydride species as an intermediate state. In the Przybyla model, Ni-signal C represents such a species. This proposal is based on the results of photolyzing the reduced enzyme (Cammack, et al., 1987, Van der Zwaan, et al., 1985). A Ni(I)-hydride and a Ni(I)-CO species, both sensitive to visible light, have been proposed as intermediates in catalysis and the reversible inhibition by CO of *C. vinosum* hydrogenase (Van der Zwaan, et al., 1986). Recently, the ^1H and ^2H Q-Band ENDOR study from *D. gigas* hydrogenase provided direct evidence for this proposal (Fan, et al., 1991). The Ni-signal A and B

represented inactive states of the enzyme. Corroborating this concept, an electron spin echo study (Chapman, et al., 1988) showed that the Ni site is inaccessible to solvent protons in the Ni-A state but is accessible in the Ni-C state. The ENDOR measurements characterized the exchangeable hydrogenic species. The Ni-C center exhibits one type of exchangeable proton that has a large hyperfine coupling. But the analysis rules out a hydride bound to the Ni-C (Fan, et al., 1991). Based on this observation, the Ni-C EPR signal species could be interpreted in terms of an equatorially coordinated hydride.

Diversity in Ni Center The structure of the Ni center may vary in different hydrogenases, based on the following facts: (1) Ni-hydrogenase from *A. vinelandii, A. eutrophus* and *B. japonicum* show only very weak or no EPR absorption from Ni, even though they contain 1 atom of Ni per enzyme. (2) Some hydrogenases, such as from *N. opaca*, contain two tightly bound Ni which are thought to be the catalytic site, in addition to loosely bound Ni atoms which aid in holding together the subunits. (3) The Ni-containing hydrogenase from the extremely thermophilic archaebacterium, *Pyrococcus furiosus*, contains one Ni atom per apparent $\alpha_2\beta_2\gamma_2$ structure. In contrast to other Ni-containing hydrogenases, it preferentially catalyzes H_2 evolution. No EPR signal characteristic of Ni was detected. The data suggest that H_2 catalysis may not occur at the Ni (or Fe-S) center (Adams, 1990a).

Role of Each Subunit

One role of the polypeptide subunits of hydrogenases is to provide ligands for holding the reactive metal centers that were discussed above. Also, the polypeptide could play roles in maintaining the correct conformation for binding of electron donors and acceptors for electron transfer. But the question naturally raised is how these various prosthetic groups distribute among the subunits of multisubunit hydrogenases and which subunit contains the site for H_2 activation. As to these questions, little is known. The

monomeric hydrogenases from *C. pasteurianum, M. elsdenii, D. desulfuricans* (ATCC), *C. vinosum, M. barkeri* and *M. vannielli* all must contain their respective prosthetic groups within a single subunit.

In the tetrameric hydrogenases isolated from *N. opaca* and *A. eutrophus* H16, four non-identical subunits are arranged as two dimers. One dimer (64 and 31 kDa) contains the diaphorase activity and 1 FMN and 10 Fe; the other dimer (56 and 27 kDa) has the hydrogenase activity and contains 2 Ni and 4 Fe (Schneider, et al., 1984). The remaining two nickels are postulated to be positioned between the two dimers. The soluble, tetrameric hydrogenase from *A. eutrophus* is similar to the hydrogenase from *N. opaca* (Hornhardt, et al., 1986, Schneider and Piechulla, 1986). From *A eutrophus*, a mutant hydrogenase was purified (Hornhardt, et al., 1986) which contained a single polypeptide with a molecular weight of 57 kDa. This polypeptide reacts with antibodies to the large subunit of the NAD^+-(NiFe) hydrogenase but failed to cross react with antibodies to the small subunit. This enzyme lacked FAD and did not reduce NAD^+, however, it did exhibit activity with artificial electron donors and acceptors although this activity was considerably less than that observed with wild-type enzyme. This large subunit has been sequenced and shown to have the nickel motif (Tran-Betcke, et al., 1990). This mutant, single subunit contains 0.2-1.4 nickel and 2-3 irons per mole of enzyme. Therefore, the large subunit (56 kDa) of these tetrameric enzyme contains the site for H_2 activation and Ni and one [4Fe-4S] binding site.

As to heterodimeric NiFe hydrogenases, two subunits are required for activity. In earlier purifications of some hydrogenases from *B. japonicum* and *A. vinelandii*, the large subunit was predominant on SDS-PAGE. These isolated hydrogenases have less activity than those isolated later on, which were shown to have two subunits (See above discussion). Recently, several site-directed mutants have been produced in the genes coding for the NiFe-dimeric hydrogenases from *E. coli* and *A. vinelandii* (Przybyla, et al.,

1992, Sayavedra-Soto and Arp, 1993). Mutations in the genes for either the large subunit or small subunit could result in the complete loss of activity. However, no conclusive evidence is available to indicate the location of the nickel and the [Fe-S] clusters. Several lines of indirect evidence suggest that the nickel is located in the large subunit: (1) The EPR spectra studies indicated that Ni reacted as redox (catalytic) site(Teixeira, et al., 1985a), (2) Introduction of ^{77}Se into NiFeSe hydrogenase from *D. baculatus* affected the Ni EPR signals (He, et al., 1989), and a putative selenocysteine codon (TGA) was found in the gene for the large subunit of the dimeric hydrogenase from *D. baculatus* (Menon, et al., 1987), suggesting that selenium and nickel in this enzyme might reside in the large subunit, (3) It was suggested that C_2H_2 binds to the H_2 activation site of the NiFe hydrogenase from *A. vinelandii* (Hyman and Arp, 1987). $^{14}C_2H_2$ associates with large subunit of this enzyme, suggesting that the H_2 activation site is located in the large subunit (Chapter II; (Sun, et al., 1992)).

Primary Structure of NiFe Hydrogenase

To date, more than 20 genes coding for hydrogenases have been sequenced. By comparison of these sequences, it has been noted that the primary sequence of Fe-only hydrogenases is very different from the NiFe hydrogenases. This lack of similarity is not surprising, because the Fe-only hydrogenases are not immunologically related to the NiFe hydrogenases (Arp, et al., 1985, Kovacs, et al., 1989). As for the NiFe hydrogenase family (including NiFe and NiFeSe hydrogenases), the primary sequences shared various degrees of homology. The NiFe hydrogenases could be classified into three subgroups. Subgroup 1 included the hydrogenase from *E. coli, A. chroococcum, A. vinelandii, B. japonicum, R. capsulata, R. gelatinosus, R. leguminosarum*, and *A. eutrophus*. Subgroup 2 included all hydrogenase from the sulfate reducing bacteria. Subgroup 3 included all NiFeSe hydrogenases.

The derived amino acid sequences of the large subunits of all nickel containing hydrogenases possess paired cysteinyl residues separated by two amino acids ($C\text{-}X_2\text{-}C$) near the amino and carboxyl termini. These structures are similar to the [Fe-S] cluster motifs found in the ferredoxin (Brushi and Guerlesquin, 1988). But in the hydrogenase case, these motifs were covered by fully conserved sequences. At amino terminal, the fully conserved sequence is R-D-P-R-D-R-X-C-G-V-C-X-X, and at carboxyl terminal the sequences is $D\text{-}P\text{-}C\text{-}X_2\text{-}C\text{-}X_2\text{-}H\text{-}V\text{-}X_{14}$. Przybyla et al. assigned these sequences as the Ni binding motifs; replacements of Cys by Ser, or Arg by Leu resulted in the completely loss of activity in hydrogenase-1 from *E. coli*. Additionally, two other conserved sequences were considered as nickel binding sites (Przybyla, et al., 1992). The first sequence (R-G-X-E) is located upstream from the amino terminal nickel motif (residues 52 -55 on the large subunit of *A. vinelandii* hydrogenase, or residues 54-58 on *E. coli* hydrogenase-1). The second sequence ($G\text{-}X_3\text{-}A\text{-}P\text{-}R\text{-}G\text{-}X_3\text{-}H$) is located upstream from the carboxyl terminal nickel motif (residues 509-520 on the large subunit of *A. vinelandii* hydrogenase, or residues 503-514 on *E. coli* hydrogenase-1). In these regions, Arg, Glu or His could supply ligands to the nickel (Przybyla, et al., 1992). However, if the entire large subunit is examined in terms of the conserved sequences, then several regions contain potential nickel binding ligands. Among these regions, a His rich region (residues 115-128 on the large subunit of *A. vinelandii* hydrogenase) could be related to the nickel coordination. The replacement of the His^{120} by Arg in *A. vinelandii* hydrogenase resulted in a dramatic decrease in H_2 oxidation and H_2 evolution activity but isotope exchange activity could not be detected in this mutant. Also, the substituted enzyme was more sensitive to inactivation than the wild type enzyme (unpublished observations). All these defects in activity and stability could be due to the unusual coordination of nickel to the protein. The small subunit contains two C-x-x-C motifs are each at the N- and C-terminals, which were shown to be required for H_2 oxidation in vivo (Sayavedra-Soto and Arp, 1993)

Brief Review of Heterodimeric, NiFe Hydrogenase from *A. Vinelandii*

Hydrogenase from *Azotobacter vinelandii* is typical of several membrane bound NiFe hydrogenase(Seefeldt and Arp, 1986). The physiological function of this hydrogenase is to consume the H_2 generated by nitrogenase during the reduction of N_2 to NH_3. This enzyme has two subunits (65kDa, and 35kDa) and contains 12.6 atoms of Fe and 1.3 atoms of Ni per mol enzyme. The ratio of Fe to Ni is 11-12. The UV-vis absorption spectrum showed the presence of [Fe-S] clusters. EPR signals apparently due to the interaction between the [4Fe-4S] and Ni and signals arising form [3Fe-4S] cluster were observed. Therefore, this enzyme most likely contain two [4Fe-4S] and one [3Fe-4S] cluster.

Like other Ni-containing hydrogenases, *A. vinelandii* hydrogenase can be purified under aerobic conditions (Sun and Arp, 1991). The catalytic properties are consistent with that of functionally related Ni-containing hydrogenases from *B. japonicum* (Arp, 1985) and *Alcaligenes latus* (Pinkwart, et al., 1983), but contrast to that of other hydrogenases from sulfate reducing bacteria and *E. coli* (Table I-1). For example, *A. vinelandii* hydrogenase has a very low rate of the back reaction, H_2 production. The H_2 scavenging function of *A. vinelandii* hydrogenase is facilitated by a low Km for H_2 (about 1 μM) and a very low rate of the back reaction, H_2 evolution.

For the *A. vinelandii* hydrogenase, several inhibitors have been characterized. (1) Dioxygen: O_2 inhibits the hydrogenase if H_2 is present along with it. Otherwise, O_2 irreversibly inactivates the hydrogenase (Seefeldt, et al., 1986). (2) Cyanide: HCN irreversibly inactivates the oxidized form of the hydrogenase. Upon inactivation, cyanide binds to the enzyme with a stoichiometry of 1.7 mol per mol of enzyme (Seefeldt and Arp, 1989). (3) Nitric oxide: *A. vinelandii* hydrogenase is very sensitive to inactivation

by nitric oxide. It was supposed that the nitric oxide could interact with the [Fe-S] clusters (Hyman and Arp, 1991). (4) Acetylene: Acetylene has been characterized as a slow, tight-binding inhibitor of the *A. vinelandii* hydrogenase. Because dihydrogen and CO are competitors for acetylene inhibition, the acetylene is supposed to interact with the Ni site of the *A. vinelandii* hydrogenase (Hyman and Arp, 1987). This thesis will further characterize the inhibitor C_2H_2 and Copper (CuII).

The genes coding for the large (*hoxG*) and small (*hoxK*) subunit of *A. vinelandii* hydrogenase were sequenced (Menon, et al., 1990). The sequences show strong similarity to genes for functionally related hydrogenases from *B. japonicum* (Sayavedra-Soto, et al., 1988) and *R. capsulatus* (Leclerc, et al., 1988), but they have less similarity to the genes for hydrogenases from *D. gigas* (Li, et al., 1987) and *D. baculatus* (Menon, et al., 1987). Furthermore, fifteen genes have now been identified in what appears to be a single hydrogenase operon. The *hoxK* is the first gene, followed by *hoxG, hoxZ, hoxM, hoxL, hoxO, hoxQ, hoxR, hoxT and hox10 ....hox15* (Chen and Mortenson, 1992a, Chen and Mortenson, 1992b). The roles of the additional genes are unknown. HoxZ is related to electron transfer from hydrogenase to the electron transport chain (Sayavedrasoto and Arp, 1992). Several site directed mutants have been isolated. The mutation in the C-x-x-C motifs of small subunit has been shown to effect the H_2 oxidation. Another interesting mutant is His^{120} replaced by Arg. It lost the ability to perform the isotope exchange reaction (see above).

References

Adams, M. W. W. (1990a) *FEMS Microbiology Reviews 75*, 219-238.

Adams, M. W. W. (1990b) *Biochim. Biophys. Acta 1020*, 115-145.

Adams, M. W. W. & Hall, D. O. (1979) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 159-169, Goltze, K. G., Gottingen.

Adams, M. W. W., Jin, S. L. C., Chem, J. S. & Mortenson, L. E. (1986) *Biochim. Biophys. Acta 869*, 37-47.

Adams, M. W. W. & Mortenson, L. E. (1984) *J. Biol. Chem. 259*, 7045-7055.

Adams, M. W. W., Mortenson, L. E. & Chen, J. S. (1981) *Biochim. Biophys. Acta. 594*, 105-176.

Albracht, S. P. J., Albrecht-Ellmer, K. J., Schmedding, D. J. M. & Slater, E. C. (1982a) *Biochim. Biophys. Acta 681*, 330-334.

Albracht, S. P. J., Graf, E. G. & Thauer, R. K. (1982b) *FEBS Lett. 140*, 311-314.

Albracht, S. P. J., Kalkman, M. L. & Slater, E. C. (1983) *Biochim. Biophys. Acta 724*, 309-316.

Albracht, S. P. J., Van der Zwaan, J. W. & Fontijn, R. D. (1984) *Biochim. Biophys. Acta 766*, 245-258.

Arp, D. J. (1985) *Arch. Biochem. Biophys. 237*, 504-512.

Arp, D. J. & Burris, R. H. (1979) *Biochim. Biophys. Acta 570*, 221-230.

Arp, D. J., McCollum, L. C. & Seefeldt, L. C. (1985) *J. Bacteriol. 163*, 15-20.

Ballantine, S. P. & Boxer, D. H. (1986) *Eur. J. Biochem. 156*, 277-284.

Bartha, R. & Ordal, E. J. (1965) *J. Bacteriol. 89*, 1015-1019.

Bell, S. H., Dickson, D. P. E., Rieder, R., Cammack, R., Patil, D. S., Hall, D. O. & Rao, K. K. (1984) *Eur. J. Biochem. 145*, 645-651.

Boursier, M. A., Hanus, F. J., Papen, H., Becker, M. M., Russell, S. A. & Evans, H. J. (1988) *J. Bacteriol. 170*, 5594-5600.

Bowien, B. & Schlegel, H. G. (1981) *Ann. Rev. Microbiol. 35*, 405452.

Brushi, M. & Guerlesquin, F. (1988) *FEMS Microbiol. Rev. 54*, 155-176.

Buchanan, B. B., Lovenberg, W. & Rabinowitz, J. C. (1963) *Proc. Natl. Acad. Sci. USA 49*, 345-353.

Cammack, R., Bagyinka, C. & Kovacs, K. L. (1989) *Eur. J. Biochem. 182*, 357-362.

Cammack, R., Fernandez, V. M. & Schneider, K. (1988) in *Bioinorganic Chemistry of Nickel* (J. R. Lancaster, Ed.) pp 167-190, VCH Publishers, New York.

Cammack, R., Fernandez, V. M. & Schneider, K. (1986) *Biochimie 68*, 85-91.

Cammack, R., Kovacs, K. L., McCracken, J. & Peisach, J. (1989) *Eur. J. Biochem. 182*, 363-366.

Cammack, R., Patil, D., Aguirre, R. & Hatchikian, E. C. (1982) *FEBS Lett. 142*, 289-292.

Cammack, R., Patil, D. S., Hatchikian, E. C. & Fernandez, V. M. (1987) *Biochim. Biophys. Acta 912*, 98-109.

Carney, M. J., Papaefthymiou, G. C., Spartalian, K., Frankel, R. B. & Holm, R. H. (1988a) *J. Am. Chem. Soc. 110*,

Carney, M. J., Papaefthymiou, G. C., Whitener, M. A., Spartalian, K., Frankel, R. B. & Holm, R. H. (1988b) *Inorg. Chem. 27*, 346-352.

Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. & Harrison, P. R. (1986) *EMBO J. 5*, 1221-1227.

Chapman, A., Cammack, R., Hatchikian, C. E., McCracken, J. & Peisach, J. (1988) *FEBS Lett. 242*, 134-138.

Chen, J. C. & Mortenson, L. E. (1992a) *Biochimica et Biophysica Acta 1131*, 122-124.

Chen, J. C. & Mortenson, L. E. (1992b) *Biochimica et Biophysica Acta 1131*, 199-202.

Colbeau, A. & Vignais, P. M. (1983) *Biochim. Biophys. Acta 748*, 128-138.

Czchowski, M. H., He, S. H., Nacro, M., Der Vartanian, D. V., Peck, H. D. J. & LeGall, J. (1984) *Biochem. Biophys. Res. Commun. 125*, 1025-1035.

de Saussure, T. (1839) *Mem. Soc. Phys. Hist. Nat. Geneve. 8*, 163.

Evans, H. J., Russell, S. A., Hanus, F. J., Papen, H., Sayavedra Soto, L., Zuber, M. & Boursier, P. (1988) in *Nitrogen Fixation: Hundred Years After* (H. Bothe, F. J. de Bruijn and W. E. Newton, Ed.) pp Gustav Fischer, Stuttgart. New York.

Fan, C. L., Gorst, C. M., Ragsdale, S. W. & Hoffman, B. M. (1991) *Biochemistry 30*, 431-435.

Farkas, A., Farkas, L. & Yudkin, J. (1934) *Proc. R. Soc. 115*, 373-379.

Fauque, G., Peck, H. D. J., Moura, J. J. G., Huynh, B. H., Berlier, Y., DerVartanian, D. V., Teixeira, M., Przybyla, A. E., Lespinat, P. A., Moura, I. & LeGall, J. (1988) *FEMS Microbiology Reviews 54*, 299-344.

Fauque, G., Teixeira, M., Moura, I., Lespinat, P. A., Xavier, A. V., Der Vartanian, D. V., Peck, H. D. J., LeGall, J. & Moura, J. J. G. (1984) *Eur. J. Biochem. 142*, 21-28.

Fernandez, V. M., Hatchikian, E. C., Patil, D. S. & Cammack, R. (1986) *Biochim. Biophys. Acta 883*, 145-154.

Fox, J. A., Livingston, D. J., Orme-Johnson, W. H. & Walsh, C. T. (1987) *Biochemsitry 26*, 4219-4227.

Francis, K., Patel, P., Wendt, J. G. & Shanmugan, K. T. (1990) *J. Bacteriol. 172*, 5750-5757.

Friedrich, C. G., Schneider, K. & Friedrich, B. (1982) *J. Bacteriol. 152*, 42-48.

Gogotov, I. N. (1986) *Biochimie 68*, 181-187.

Graf, E. G. & Thauer, R. K. (1981) *FEBS Lett. 115*, 285-288.

Harker, A. R., Xu, L., Hanus, F. J. & Evans, H. J. (1984) *J. Bacteriol. 159*, 850-856.

Hatchikian, E. C., Bruschi, M. & J., L. (1978) *Biochem. Biophys. Res. Commun. 82*, 451-461.

Hausinger, R. P. (1987) *Microbiological Reviews 51*, 22-42.

He, S. H., Teixeira, M., LeGall, J., Patil, D. S., Der Vartanian, D. V., Huynh, B. H. & Perk, H. D. J. (1989) *J. Biol. Chem. 264*, 2678-2682.

Hoberman, H. D. & Rittenberg, D. (1943) *J. Biol. Chem. 147*, 211-227.

Hornhardt, S., Schneider, K. & Schlegel, H. G. (1986) *Biochimie 68*, 15-24.

Houchins, J. P. (1984) *Biochim. Biophys. Acta 768*, 227-255.

Huang, Y.-H., Park, J.-B., Adams, M. W. W. & Johnson, M. K. (1993) *Inorg. Chem. 32*, 375-376.

Huynh, B. H., Patil, D. S., Moura, I., Teixeira, M., Moura, J. J. G., DerVartanian, D. V., Czechowski, M. H., Prickrill, B. C., Peck, H. D. J. & LeGall, J. (1987) *J. Biol. Chem. 262*, 795-800.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1987) *Biochemistry 26*, 6447-6454.

Jin, S. L. C., Blanchard, D. K. & Chem, J. S. (1983) *Biochim. Biophys. Acta 748*, 8-20.

Joklik, W. K. (1950) *Aust. J. Exp. Biol. Med. Sci. 28*, 331-338.

Juszczak, A., Aono, S. & Adams, M. W. W. (1991) *Journal of Biological Chemistry 266*, 13834-13841.

Kaserer, H. (1906) *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 2*, 681.

Koch, H. G., Kern, M. & Klemme, J. H. (1992) *FEMS Microbiology Letters 91*, 193-197.

Kojima, N., Fox, J. A., Hausinger, R. P., Daniels, L., Orme-Johnson, W. H. & Walsh, C. T. (1983) *Proc. Natl. Acad. Sci. USA 80*, 378-382.

Kovacs, K. L., Seefeldt, L. C., Tigyi, G., Doyle, C. M., Mortenson, L. E. & Arp, D. J. (1989) *J. Bacteriol. 171*, 430-435.

Kow, Y. W. & Burris, R. H. (1984) *J. Bacteriol. 159*, 564-569.

Krasna, A. I. & Rittenberg, D. (1954) *J. Am. Chem. Soc. 76*, 3015-3020.

Kruger, H. J., Huynh, B. H., Ljungdahl, P. O., Xavier, A. V., Der Vartanian, D. V., Moura, I., Peck, H. D. J., Teixeira, M., Moura, J. J. G. & LeGall, J. (1982) *J. Biol. Chem. 257*, 14620-14623.

Kumar, M., Day, R. O., Colpas, G. J. & Maroney, M. J. (1989) *J. Am. Chem. Soc. 111*, 5974-5976.

Lalla-Maharajh, W. V., Hall, D. O., Cammack, R., Rao, K. K. & LeGall, J. (1983) *Biochem. J. 209*, 445-454.

Lancaster, J. R. J. (1982) *Science 216*, 1324-1325.

Lancaster, J. R. J. (1980) *FEBS Lett. 115*, 285-288.

Leclerc, M., Colbeau, A., Cauvin, B. & Vignais, M. (1988) *Mol. Gen. Genetics 214*, 97-107.

LeGall, J., Ljungdahl, P. O., Moura, I., Peck, H. D. J., Xavier, A., Moura, J. J. G., Teixeira, M., Huynh, B. H. & Der Vartanian, D. V. (1982) *Biochem. Biophys. Res. Commun. 106*, 610-615.

Lespinat, P. A., Berlier, Y., Fauque, G., Czechowski, M. H., Dimon, B. & LeGall, J. (1986) *Biochimie 68*, 55-61.

Li, C., Peck Jr., H. D., LeGall, J. & Przybyla, A. E. (1987) *DNA 6*, 539-551.

Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. H. & Munck, E. (1985) *J. Biol. Chem. 260*, 11160-11173.

Lindahl, P. A., Gorelick, N. J., Munck, E. & Orem-Johnson, W. H. (1987) *J. Biol. Chem. 262*, 14945-14953.

Lindahl, P. A., Kojima, N., Hausinger, R. P., Fox, J. A., Jeo, B. K., Walsh, C. T. & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 3062-3064.

Lissolo, T., Choi, E. S., LeGall, J. & Peck, H. D. J. (1986) *Biochem. Biophys. Res. Commun. 139*, 701-708.

Livingston, D. J., Fox, J. A., Orme-Johnson, W. H. & Walsh, C. T. (1987) *Biochemistry 26*, 4228-4237.

Lovenberg, W., Buchanan, B. B. & Rabinowitz, J. C. (1963) *J. Biol. Chem. 238*, 3899-3913.

Maier, T., Jacobi, A., Sauter, M. & Bock, A. (1993) *Journal of Bacteriology 175*, 630-635.

Mayhew, S. G., Van Dijk, C. & Van der Westen, H. M. (1978) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 125-140, Erich Goltze, Gottingen, FRG.

Menon, A. L., Stults, L. W., Robson, R. L. & Mortenson, L. E. (1990) *Gene 96*, 67-74.

Menon, N. K., Peck, H. D. J., LeGall, J. & Przybyla, A. E. (1988) *J. Bacteriol. 170*, 4429.

Menon, N. K., Peck Jr., H. D., LeGall, J. & Przybyla, A. E. (1987) *J. Bacteriol. 169*, 5401-5407.

Menon, N. K., Robbins, J., Peck Jr., H. D., Chatelus, C. Y., Choi, E.-S. & Przybyla, A. E. (1990) *J. Bacteriol. 172*, 1969-1977.

Menon, N. K., Robbins, J., Wendt, J. C., Shanmugam, K. T. & Przybyla, A. E. (1991) *Journal of Bacteriology 173*, 4851-4861.

Mortenson, L. E., Valentine, R. C. & Carnahan, J. E. (1962) *Biochem. Biophys. Res. Commun. 7*, 448-452.

Muth, E., Morschel, E. & Klein, A. (1987) *Eur. J. Biochem. 109*, 571-577.

Nakos, G. & Mortenson, L. E. (1971a) *Biochim. Biophys. Acta 227*, 576-583.

Nakos, G. & Mortenson, L. E. (1971b) *Biochemistry 10*, 2442-2449.

Niviere, V., Forget, N., Gayda, J. P. & Hatchikian, E. C. (1986) *Biochem. Biophys. Res. Commun. 139*, 658-665.

Peck, H. D. J. & Gest, H. (1957) *J. Bacteriol. 73*, 569-580.

Pinkwart, M., Schneider, K. & Schlegel, H. G. (1983) *Biochim. Biophys. Acta. 745*, 267-278.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1992) *FEMS Microbiology Reviews 88*, 109-135.

Reeve, J. N. & Beckler, G. S. (1990) *FEMS Microbiol. Revs. 87*, 425-430.

Rieder, R., Cammack, R. & Hall, D. O. (1984) *Eur. J. Biochem. 145*, 637-643.

Ruhland, W. (1922) *Ber. Botan. Ges. 40*, 180-184.

Sadana, J. C. & Jagannathan, V. (1956) *Biochim. Biophys. Acta 19*, 446-452.

Sadana, J. C. & Rittenberg, D. (1963) *Proc. Natl. Acad. Sci. USA 50*, 900-904.

Sawers, R. G. & Boxer, D. H. (1986) *Eur. J. Biochem. 156*, 265-275.

Sayavedra-Soto, L. A. & Arp, D. J. (1993) *J. Bacteriol. 175*, 3414-3421.

Sayavedrasoto, L. A. & Arp, D. J. (1992) *Journal of Bacteriology 174*, 5295-5301.

Sayavedra-Soto, L. A., Powell, G. A., Evans, H. J. & Morris, R. O. (1988) *Proc. Natl. Acad. Sci. USA 85*, 8395-8399.

Schink, B. & Schlegel, H. G. (1979) *Biochim. Biophys. Acta. 567*, 315-324.

Schneider, K. & Cammack, R. (1978) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 221-234, Erich Goltze, Gottingen, FRG.

Schneider, K., Cammack, R. & Schlegel, H. G. (1984) *Eur. J. Biochem. 142*, 75-84.

Schneider, K. & Piechulla, B. (1986) *Biochimie 68*, 5-13.

Schneider, K., Patil, D. S. & Cammack, R. (1983) *Biochim. Biophys. Acta 748*, 353-361.

Scott, R. A., Wallin, S. A., Czechowski, M., Der Vartanian, D. V., Le Gall, J., Peck, H. D. J. & Moura, I. (1984) *J. Am. Chem. Soc. 106*, 6864-6865.

Seefeldt, L. C. (1989) Ph. D thesis, University of California, Riverside.

Seefeldt, L. C. & Arp, D. J. (1989) *J. Bacteriol. 171*, 3298-3303.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Seefeldt, L. C., Fox, C. A. & Arp, D. J. (1986) *J. Biol. Chem. 261*, 10688-10694.

Seefeldt, L. C., McCollum, L. C., Doyle, C. M. & Arp, D. J. (1987) *Biochim. Biophys. Acta 914*, 299-303.

Shug, A. L., Wilson, P. W., Green, D. E. & Mahler, H. R. (1954) *J. Am. Chem. Soc. 76*, 3355-3356.

Stephenson, M. & Stickland, L. H. (1931) *Biochem. J. 25*, 205-215.

Stults, L. W., O'Hara, E. B. & Maier, R. J. (1984) *J. Bacteriol. 159*, 153-158.

Sun, J. H. & Arp, D. J. (1991) *Arch. Biochem. Biophys. 287*, 225-233.

Sun, J. H., Hyman, M. R. & Arp, D. J. (1992) *Biochemistry 31*, 3158-3165.

Tamiya, N., Kondo, Y., Kameyama, T. & Akabori, E. (1955) *J. Biochem. 42*, 613-614.

Tan, S. L., Fox, J. A., Kojima, N., Walsh, C. T. & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 3064-3066.

Teixeira, M., Fanque, G., Moura, I., Lespinat, P. A., Berlier, Y., Pickrill, B., Peck, H. D. J., Xavier, A. V., LeGall, J. & Moura, J. J. G. (1987) *Eur. J. Biochem. 167*, 47-58.

Teixeira, M., Moura, I., Fauque, G., DerVartanian, D. V., LeGall, J., Peck Jr., H. D., Moura, J. J. G. & Huynh, B. H. (1990) *Eur. J. Biochem. 189*, 381-386.

Teixeira, M., Moura, I., Xavier, A. V., Huynh, B. H., Der Vartanian, D. V., Peck, H. D., Jr., LeGall, J. & Moura, J. J. G. (1985a) *J. Biol. Chem. 260*, 8942-8950.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., Fauque, G., Pickril, B. & LeGall, J. (1985b) *Rev. Port. Quim. 27*, 194-195.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., LeGall, J., Der Vartanian, D. V., Peck, H. D., Jr. & Huynh, B. H. (1989) *J. Biol. Chem. 264*, 16435-16450.

Tigyi, G., Bagyinka, C. & Kovacs, K. C. (1986) *Biochimie 68*, 69-74.

Torres, V., Ballesteros, A. & Fernandez, V. M. (1986) *Arch Biochem. Biophys. 245*, 174-178.

Tran-Betcke, A., Warnecke, U., Boecker, C., Zaborosch, C. & Friedrich, B. (1990) *J. Bacteriol. 172*, 2920-2929.

Unden, G., Bocher, R., Knecht, J. & Kroger, A. (1982) *FEBS Lett. 145*, 230-234.

van der Werf, A. N. & Yates, M. G. (1978) in *Hydrogenases: Their catalytic activity, structure and function* (H. G. Schlegel and K. Schneider, Ed.) pp 307-326, Erich Goltze KG.

Van der Westen, H. M., Mayhew, S. G. & Veeger, C. (1978) *FEBS Lett. 86*, 122-126.

Van derZwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Mul, P. (1987) *Eur. J. Biochem. 169*, 377-384.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Roelofs (1986) *Biochim. Biophys. Acta 872*, 208-215.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Slater, E. C. (1985) *FEBS Lett. 179*, 271-277.

Van Heerikhuizen, H., Albracht, S. P. J., Slater, E. C. & Van Rheenen, P. S. (1981) *Biochim. Biophys. Acta 657*, 26-39.

Voordouw, G. & Brenner, S. (1985) *Eur. J. Biochem. 148*, 515-520.

Walsh, C. T. & Orme-Johnson, W. H. (1987) *Biochemistry 26*, 4901-4906.

Waring, W. S. & Werkman, C. H. (1944) *Arch. Biochem. 4*, 75-87.

Wendel, A., Pilz, W., Ladenstein, R., Sawatzki, G. & Weser, U. (1975) *Biochim. Biophys. Acta 377*, 211-215.

Yamazaki, S. (1982) *J. Biol. Chem. 257*, 7926-7929.

Yates, M. G., Ford, C. M., Tibelius, K. H., Campbell, F., Arp, D. J. & Seefeldt, L. C. (1988) in *Nitrogen Fixation: Hundred Years After* (H. Bothe, F. J. de Bruijn and W. E. Newton, Ed.) pp 263-269, Gustav Fischer, New York.

Zinoni, F., Birkmann, A., Stadtman, T. C. & Bock, A. (1986) *Proc. Natl. Acad. Sci. USA 83*, 4650-4654.

Zirngibl, C., Vandongen, W., Schworer, B., Vonbunau, R., Richter, M., Klein, A. & Thauer, R. K. (1992) *European Journal of Biochemistry 208*, 511-520.

Zorin, N. A. (1986) *Biochimie 68*, 97-101.

CHAPTER II

C_2H_2 INHIBITION OF *AZOTOBACTER VINELANDII* HYDROGENASE: C_2H_2 BINDS TIGHTLY TO THE LARGE SUBUNIT[1, 2]

by

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Running Title: C_2H_2 Inhibition of *Azotobacter* Hydrogenase

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Contributions

Jin-hua Sun participated in developing the experimental design, performing the experiments, analyzing the data and calculating the results. Dr. Michael R. Hyman participated in developing the techniques utilized to perform this study and editing the manuscript. Dr. Daniel Arp participated in the experimental design, supervising progress of study and editing the manuscript.

Abbreviations

EDTA; ethylenediaminetetraacetic acid

EPR; electron paramagnetic resonance

EXAFS; extended X-ray absorption fine structure

SDS; sodium dodecylsulfate

SDS-PAGE; sodium dodecylsulfate-polyacrylamide gel electrophoresis

TCA; trichloroacetic acid

Tris; tris(hydroxymethyl)aminomethane

Abstract

Acetylene is a slow-binding inhibitor of the Ni- and Fe-containing dimeric hydrogenase isolated from *Azotobacter vinelandii*. Acetylene was released from hydrogenase during the recovery from inhibition. This indicates that no transformation of acetylene to another compound occurred as a result of the interaction with hydrogenase. However, the release of C_2H_2 proceeds more rapidly than the recovery of activity which indicates that release of C_2H_2 is not sufficient for recovery of activity. Acetylene binds tightly to native hydrogenase; hydrogenase and radioactivity coelute from a gel permeation column following inhibition with $^{14}C_2H_2$. Acetylene, or a derivative, remains bound to the large, 65,000 MW subunit (and not to the small, 35,000 MW subunit) of hydrogenase following denaturation as evidenced by SDS-PAGE and fluorography of $^{14}C_2H_2$-inhibited hydrogenase. This result suggests that C_2H_2, and by analogy, H_2, bind to and are activated by the large subunit of this dimeric hydrogenase. Radioactivity is lost from $^{14}C_2H_2$-inhibited protein during recovery. The inhibition is remarkably specific for C_2H_2-- propyne, butyne and ethylene are not inhibitors.

Introduction

The nitrogen-fixing bacterium, *Azotobacter vinelandii*, expresses a single, membrane-bound hydrogenase. The physiological function of this enzyme is to oxidize the H_2 produced by nitrogenase during the reduction of N_2 to NH_3. *A. vinelandii* hydrogenase efficiently scavenges the H_2 produced *in situ* by nitrogenase. This efficiency is facilitated by the high affinity for H_2 (K_m near 1 μM) and the low rate of the back reaction (production of H_2) (Seefeldt and Arp, 1986; Kow and Burris, 1984). As isolated, hydrogenase from *A. vinelandii* consists of two nonidentical subunits of about 65,000 and 35,000 molecular weight which are present in a one to one ratio to give a native molecular weight near 100,000. The enzyme also contains Ni and Fe in a 1 to 10-11 ratio (Seefeldt and Arp, 1986). EPR and UV-vis spectroscopy indicate that the Fe is present in [Fe-S] centers, though the exact number and type are not known (L.C. Seefeldt, Ph.D. thesis, University of California-Riverside, 1989).

Hydrogenase from *A. vinelandii* is typical of a number of hydrogenases isolated from physiologically distinct groups of microorganisms. For example, hydrogenases isolated from *Rhodobacter capsulatus*, *Alcaligenes eutrophus*, *Escherichia coli*, *Desulfovibrio gigas*, *Desulfovibrio baculatus*, *Thiocapsa roseopersicina* and *Bradyrhizobium japonicum* all have similar subunit compositions and contain Ni and FeS centers (Przybyla et al., 1991). The similarity among these NiFe hydrogenases is further reflected in their cross-reactivity to antibodies raised against individual hydrogenases (Kovacs et al., 1989). The structural genes coding for several of these NiFe hydrogenases have been sequenced and they reveal a strong conservation in the locations of a number of amino acids, especially cysteines (the likely ligands to the FeS centers) and histidines as well as the amino acids flanking these cysteines and histidines (Przybyla et al., 1991).

It is of interest to determine the roles of each of the subunits in the oxidation of H_2 by these hydrogenases as well as the location and function of the metal centers. Nickel is apparently bound to the large subunit of the *D. baculatus* hydrogenase. ^{77}Se-EPR (He et al., 1989b) and EXAFS (Eidsness et al., 1989) have revealed an interaction of the Ni with Se which is found on selenocysteine (amino acid residue #493 on the large subunit: Voordouw et al., 1989). This selenocysteine is replaced by a conserved cysteine in other NiFe hydrogenases, leading to the suggestion that this cysteine binds Ni in these hydrogenases (Przybyla et al., 1991). However, analysis by proton induced X-ray emission spectroscopy of the metal content of the subunits of *T. roseopersicina* hydrogenase following separation of the subunits by SDS-PAGE indicated that the Ni was located exclusively on the small subunit, while the remaining Fe was located on the large subunit (Bagyinka et al., 1989). The subunit distribution of the FeS centers is not known but the presence of several conserved cysteines in the small subunit (Przybyla et al., 1991) suggests that at least some of the FeS centers are located in the small subunit.

Inhibitors provide a means of investigating the mechanism of H_2 oxidation by hydrogenase and of probing the role of the metal centers in catalysis. A number of inhibitors of *A. vinelandii* hydrogenase have now been characterized, including O_2 (Seefeldt and Arp, 1989b), CN^- (Seefeldt and Arp, 1989a) and NO (Hyman and Arp, 1991). This manuscript deals with the inhibitor, C_2H_2. Smith et al. (Smith et al., 1976) first recognized the ability of C_2H_2 to inhibit hydrogenase in intact *Azotobacter chroococcum* cells. Yates and coworkers (van der Werf and Yates, 1978) demonstrated that the inhibition required preincubation of hydrogenase in the absence of H_2 and that the inhibition was reversible. Hyman and Arp (Hyman and Arp, 1987a) provided a thorough characterization of the kinetic mechanism of C_2H_2 inhibition. Acetylene is a slow-binding, active-site directed inhibitor of *A. vinelandii* hydrogenase. H_2 is a potent and competitive protectant against inhibition by C_2H_2. He et al. (He et al., 1989a) showed that the NiFe hydrogenase of *D. gigas* and the NiFeSe hydrogenase of *D.*

baculatus are inhibited by C_2H_2 while the "Fe-only" hydrogenase of *Desulfovibrio vulgaris* is not inhibited by C_2H_2. This supported the idea that C_2H_2 reacted with Ni in NiFe hydrogenases (He et al., 1989a; Hyman and Arp, 1987a). However, Juszczak et al., (Juszczak et al., 1991) have recently described a hydrogenase isolated from the extremely thermophilic eubacterium, *Thermotoga maritima*, that does not appear to contain Ni but is inhibited by C_2H_2.

Despite the interest in C_2H_2 as an inhibitor of hydrogenases, several fundamental questions regarding the mechanism of C_2H_2 inhibition remain. For example, it has not been demonstrated that C_2H_2 remains bound to hydrogenase following inhibition, nor has it been demonstrated that C_2H_2, rather than a derivative of C_2H_2, is released during recovery from C_2H_2 inhibition. We have proposed that C_2H_2 might act as an analogue of H_2 (Hyman and Arp, 1987a). This raises the possibility that C_2H_2, like H_2, is activated by hydrogenase and transformed to another compound. Perhaps the transformed compound is the actual inhibitor. Alternatively, the transformed C_2H_2 might be released from the enzyme leaving behind an inactive hydrogenase or the transformed C_2H_2 could remain bound while hydrogenase is inhibited and then be released as C_2H_2 during recovery. In this work, we have further investigated the mechanism of C_2H_2 inhibition of *A. vinelandii* hydrogenase. The inhibition was specific for C_2H_2 and no transformation of C_2H_2 was observed. Acetylene (or a derivative) was bound to the enzyme during the inhibition and was released prior to recovery of activity. Acetylene (or a derivative) remained bound to the large subunit following denaturation of hydrogenase. The results provide the first biochemical evidence that C_2H_2 and, most likely H_2 as well, bind to the large subunit of this Ni-containing hydrogenase.

Materials and Methods

Materials

Residual O_2 was removed from H_2 and N_2 (>99.99% purity) by passage over a heated copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). Gas from an acetylene cylinder (99.6%) was vented until no H_2 gas was detectable by gas chromatography. Acetylene was further purified cryogenically as described (Hyman and Arp, 1987b). All electrophoresis reagents were purchased from Schwaarz/Mann Biotech (Cleveland, Ohio). Nitrocellulose paper (0.45 μm) was obtained from Micro Filtration Systems (Dublin, CA). Peroxidase-conjugated goat antirabbit IgG was puchased from TAGO, Inc. (Burlingame, CA). All other reagents were obtained from Sigma (St. Louis, MO).

Purification of *A. vinelandii* Hydrogenase

All experiments were carried out with highly purified hydrogenase. Cells of *A. vinelandii* (strain OP) were cultured and membranes were prepared as described (Seefeldt and Arp, 1989b). The hydrogenase was purified from membranes as previously described (Sun and Arp, 1991). All steps were performed under anaerobic conditions and in the presence of 2 mM $Na_2S_2O_4$.

Protein Determinations

A comparison of protein concentration determinations by three different methods revealed that both the Bradford dye-binding assay (Bradford, 1976) and the biuret assay (Gornall et al., 1949) overestimated the protein concentration in solutions of highly

purified *A. vinelandii* hydrogenase by a factor of 2.2 compared to determinations of total amino acid compositions in hydrogenase hydrolysates. A similar result was observed for the Fe-only hydrogenases isolated from *Clostridium pasteurianum* (Adams et al., 1989). In this work, protein concentrations were estimated with the Bradford assay, then corrected according to the results of the total amino acid analyses. With this estimate of protein concentration, the specific activity of the purified hydrogenase was 300 units·mg protein^{-1} (pH 6.0, methylene blue assay at 30°C).

SDS-PAGE

Discontinuous vertical slab gels (10 or 12%(w/v) acrylamide; 10 x 6.0 x 0.15 cm) were prepared as described (Hathaway et al., 1979). Hydrogenase samples and molecular weight standards were mixed in equal volumes (or as indicated) with SDS-PAGE sample buffer (0.25 M Tris, 0.003% w/v bromophenol blue, 30% v/v glycerol, 6% w/v SDS, 15% v/v 2-mercaptoethanol, pH 6.8) and applied to the gel without heating. Molecular weight standards were phosphorylase b (97,400), ovalbumin (45,000), carbonic anhydrase (29,000), myoglobin (17,000), and cytochrome c (12,300). Proteins were visualized by staining with Coomassie blue.

Incubation Procedures for C_2H_2 Inhibition

Incubations of hydrogenase with C_2H_2 were carried out in shortened test-tubes (0.5 ml volume) placed in serum vials (10 ml) sealed with butyl rubber caps and crimped aluminum seals (Wheaton Scientific, Millville, NJ). The vials were evacuated and then filled with C_2H_2 (101 kPa) or a mixture of C_2H_2 and N_2. Incubations were initiated by addition of hydrogenase to the incubation tube. The final reaction mixture consisted of purified hydrogenase, 2 mM EDTA and 2 mM $Na_2S_2O_4$ in 50 mM Tris-HCl (pH 7.5).

Each vial also contained an O_2 scavenger (0.5 ml of 0.1 M $Na_2S_2O_4$ in 0.1 M Tris-HCl, pH 7.5) outside the incubation tube. At the indicated times, a sample of the enzyme was removed from the incubation tube and either assayed for hydrogenase activity or mixed with SDS-PAGE sample buffer (see above) for further analysis.

Recovery of Activity Following C_2H_2 Inhibition

To allow hydrogenase to recover from inhibition by C_2H_2, unbound C_2H_2 in the inhibition mixture was removed by repeated evacuation or, in radioactive experiments, by equilibration of the hydrogenase solution with Ar. The inhibited hydrogenase was then transferred to the inner chamber of a double-chambered vial which contained 101 kPa H_2. The outer section of the vial contained an O_2 scavenger (see above). The $Na_2S_2O_4$ concentration in the enzyme sample was raised to 4 mM by addition of $Na_2S_2O_4$ from a stock solution (0.1 M). At the indicated times, a sample of the enzyme was removed from the incubation vial and either assayed for hydrogenase activity or mixed with SDS-PAGE sample buffer (see above) for further analysis.

Hydrogenase Activity Assays

Reduction of methylene blue coupled to H_2 oxidation was determined as a measure of hydrogenase activity (Arp and Burris, 1981).

Fluorography of ^{14}C-Labeled Polypeptides

For fluorography of ^{14}C-labeled polypeptides separated by SDS-PAGE, the gels were impregnated with a scintillant (2,5-diphenyloxazole), dried, and exposed to X-ray film (Kodak XAR5) for 3-7 days at -70°C as described (Bonner and Laskey, 1974).

Western Immunoblot Analysis

The proteins in polyacrylamide gels to be analyzed by a Western immunoblot technique were electroblotted onto nitrocellulose paper with a semi-dry blotter. An enzyme-linked immunosorbent assay was performed on the nitrocellulose sheet as described (Birkett et al., 1985) with antiserum (200-fold dilution) prepared against *B. japonicum* hydrogenase large subunit or small subunit. Peroxidase-conjugated goat antirabbit antibodies were used diluted 2000-fold (Seefeldt and Arp, 1987).

$^{14}C_2H_2$ Preparation

$^{14}C_2H_2$ was synthesized from $Ba^{14}CO_3$ by a modification of a previously described method (Hyman and Arp, 1990). Briefly, 2.5 mCi $Ba^{14}CO_3$ (Specific activity = 56 mCi/mmol) was thermally fused with approximately 300 mg finely shredded Ba metal in a pyrex ignition tube. The fused material containing $Ba^{14}C_2$ was transferred to a glass serum vial (160 ml). The vial was stoppered with a butyl rubber stopper from which was suspended a strip (2 cm by 5 cm) of filter paper that had previously been impregnated with 0.2 ml of an aqueous solution of 10% (w/v) silver nitrate and allowed to dry. The hydrolysis of the BaC_2 fusion mixture was initiated by the addition of 1 ml water. After 1 hr, the vial was opened to remove the filter paper which had adsorbed the $^{14}C_2H_2$ in the form of silver acetylide. The filter paper was then transferred to a serum vial (6 ml) which contained an inner vial (0.5 ml) cemented to the inside floor. The vial was stoppered and flushed with Ar for 10 min to deoxygenate the vial. This provided an effective separation of the $^{14}C_2H_2$ from other contaminating gases. The $^{14}C_2H_2$ was subsequently released from the filter paper by the sequential additions of 1 ml of an aqueous solution of 1 M $Na_2S_2O_4$ (to reduce the silver acetylide to elemental silver and

free acetylene) and 0.2 ml of 1 N NaOH (to absorb SO_2 generated by the oxidation of $Na_2S_2O_4$).

$^{14}C_2H_2$-Binding Studies

Purified *A. vinelandii* hydrogenase (175 µg) was incubated in 60 µl of 20 mM Tris-HCl, 2 mM EDTA and 2 mM $Na_2S_2O_4$ (pH 7.5) under a gas phase of 2.8 kPa $^{14}C_2H_2$ (determined from the radioactivity in the aqueous solution equilibrated with the gas phase) and 98 kPa Ar for 24 hr which resulted in 67% inhibition of hydrogenase activity. The majority of the unbound C_2H_2 was removed by equilibration of the solution in a 10 ml vial filled with Ar. The solution was then removed and loaded onto a Sephadex G-25 column (10 cm long by 0.6 cm diameter) equilibrated with H_2-purged, 20 mM Tris-HCl, 2 mM EDTA and 2 mM $Na_2S_2O_4$ (pH 7.5). As the column was developed, fractions of approximately 100 µl were collected in N_2-filled vials. A sample (10 µl) was removed from each fraction and added to 1.5 ml of liquid scintillation counting fluid followed by counting in a Beckman LS 3801 counter in the ^{14}C window. Counting efficiency was determined to be 80%. The remainder of each fraction was injected into a activation vial (see "Recovery of activity..." above) and was incubated with 101 kPa H_2 for 50 hrs. The $^{14}C_2H_2$-binding experiment was repeated but with the inclusion of H_2 (20 kPa) during the initial incubation. The H_2 prevented C_2H_2 inhibition (Hyman and Arp, 1987a); the sample retained 97% of the initial activity during the incubation in the presence of C_2H_2.

C_2D_2 Preparation

Deuterated acetylene (C_2D_2) was generated by adding 10 ml D_2O (99% purity) to 3 g CaC_2 in a stoppered side-armed flask (50 ml). The resulting gas was collected in a

cryogenic gas purification vessel (Hyman and Arp, 1987b) immersed in liquid N_2. After the hydrolysis of the CaC_2 was complete, the collection vessel was evacuated to remove non-condensed contaminating gases. The collection vessel was then allowed to warm and the condensed C_2D_2 sublimed and filled evacuated serum vials connected to the collection vessel. This method of acetylene generation did not make use of the previously described H_2SO_4 trap (Hyman and Arp, 1987b) so as to eliminate proton exchange between C_2D_2 and the acid. Protonated acetylene (C_2H_2) used for rate comparisons was generated in exactly the same way except that D_2O was replaced with H_2O.

Results

Acetylene Is Released from Hydrogenase during Recovery from Inhibition

Previous studies demonstrated that inhibition of hydrogenases by C_2H_2 is time-dependent and reversible (van der Werf and Yates, 1978; Hyman and Arp, 1987a). However, these studies did not consider the possibility that C_2H_2 is transformed by hydrogenase to another compound during the inhibition. To test this possibility, the reaction mixtures following inhibition of hydrogenase with C_2H_2 were analyzed by gas chromatography for potential reaction products. No evidence of the production of ethylene, ethane, methane or acetaldehyde was detected. Sufficient quantities of hydrogenase (50-100 pmol) were used in these experiments that even a single catalytic turnover event by each hydrogenase molecule would have been detected. These results suggested that C_2H_2 was not converted to another compound by hydrogenase.

To confirm that C_2H_2 was not transformed by hydrogenase, a hydrogenase sample was inhibited with C_2H_2, the unbound C_2H_2 was removed and the release of C_2H_2 during recovery of activity was determined. Hydrogenase was inhibited with C_2H_2 (50 kPa, 20 hr) until the activity had decreased to less than 1% of the original activity. Unbound C_2H_2 was then removed from the hydrogenase solution by evacuation and equilibration with Ar followed by passage of the enzyme through a gel permeation column. The protein-containing fractions were then combined and incubated under H_2. Activity slowly recovered during the next 70 hours to 100 % of the original value (Fig. 1). During this time, samples of the gas phase were removed and analyzed by gas chromatography. The results (Fig. 1) revealed that a gaseous compound that comigrated with C_2H_2 was released during the recovery of activity from C_2H_2 inhibition. To further confirm the identity of this compound as C_2H_2, $AgNO_3$ (which complexes selectively

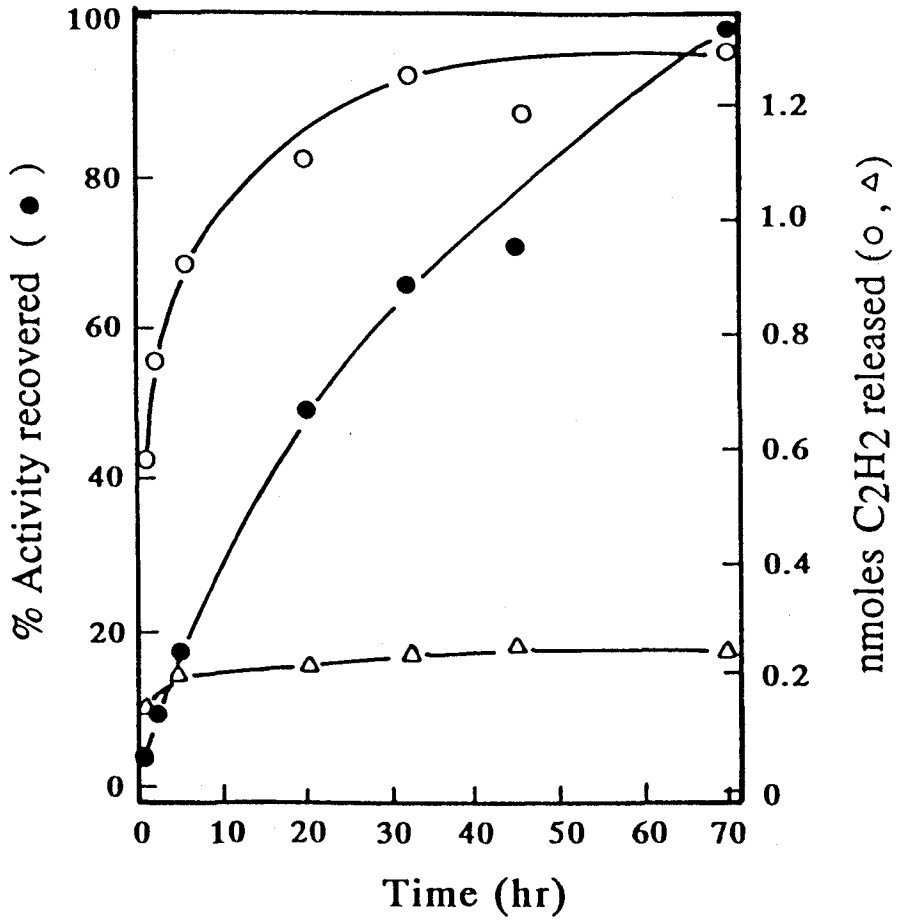


Figure II. 1. Release of C_2H_2 from and Recovery of Activity by C_2H_2-inhibited Hydrogenase. C_2H_2-inhibited hydrogenase (50 µl, 2.54 mg/ml protein) was passed through a Sephdex G-25 column and eluted with 50 mM Tris-HCl (pH 7.5) under Ar to remove the unbound C_2H_2. Eluted fractions which contained protein were immediately combined, evacuated for 2 min and then incubated under 101 kPa H_2. At the indicated incubation times, a gas sample (0.2 ml) was removed and the amount of C_2H_2 was quantified by gas chromatography (o). An additional sample (1 µl) was removed for determination of hydrogenase activity (•). The experiment was repeated, except that the hydrogenase was incubated in the presence of C_2H_2 (99 kPa) plus H_2 (2 kPa) during the initial inhibition phase and activity was retained. Gas samples (0.2 ml) were removed during a subsequent incubation and the amount of C_2H_2 was quantified by gas chromatography (Δ).

with n-terminal alkynes) was added to the reaction vials and this resulted in the disappearance of the compound that coeluted with C_2H_2. A hydrogenase sample incubated in the presence of H_2 and C_2H_2 was not inhibited and maintained full activity throughout the recovery period. Only a small amount of C_2H_2 was released from this sample during the recovery period (Fig. 1). For the hydrogenase sample inhibited with C_2H_2, the amount of C_2H_2 released into the gas phase was 1.29 nmol, which compares to the 1.27 nmol of hydrogenase used in the experiment. It is noteworthy that the kinetics of release of C_2H_2 into the gas phase did not correspond with the recovery of activity, rather C_2H_2 release proceeded more rapidly than recovery of activity. For example, most of the C_2H_2 (89 %) had been released within 20 hours, while only a 47% increase in activity was observed during this time. This observation may also provide an explanation for the amount of gaseous C_2H_2 present in the vial at time taken as t=0 (note that this C_2H_2 must have coeluted with the hydrogenase and that the quantity was substantially greater than in the uninhibited control) . Apparently, a substantial amount of C_2H_2 was released from hydrogenase during the approximately 20 min following the gel permeation column and preceding the removal of the first sample for gas chromatography.

Acetylene Binds tightly to Hydrogenase

The results of the experiment described above (Fig. 1) indicate that C_2H_2 (or a derivative) binds tightly to hydrogenase during inhibition. To directly demonstrate the binding of C_2H_2, or a derivative of C_2H_2, to hydrogenase, we inhibited hydrogenase with $^{14}C_2H_2$ and then quantified the radioactivity associated with the hydrogenase. This experiment required consideration of a number of technical limitations. For example, it was necessary to synthesize the $^{14}C_2H_2$ and to remove interfering contaminants such as H_2. The low association rate constant for binding of C_2H_2 to hydrogenase indicates an exceptionally sluggish interaction (Schloss, 1988), which demands that high partial

pressures of C_2H_2 (50-101 kPa) be used in order to obtain rapid and complete inhibitions (> 90% inhibition in < 1hr). However, it is not practical to use high concentrations of purified $^{14}C_2H_2$ of high specific activity. Therefore, the inhibitions took place in low concentrations of $^{14}C_2H_2$ (2-5 kPa) for long periods of time (typically 24 hr) and did not proceed to completion. Finally, all manipulations required strictly anaerobic conditions.

When hydrogenase was incubated in the presence of $^{14}C_2H_2$ (2.8 kPa) for 24 hr, the activity was inhibited by 67%. Following the removal of the majority of the unbound $^{14}C_2H_2$ from the enzyme solution by equilibration with 100 volumes of Ar, the enzyme solution was passed through a gel permeation column to separate the remaining unbound $^{14}C_2H_2$ from the protein. Determinations of the radioactivity in the column fractions revealed that ^{14}C from $^{14}C_2H_2$ co-eluted with hydrogenase activity (Fig. 2). When H_2 was included during the initial incubation with $^{14}C_2H_2$, the sample retained activity and the amount of radioactivity which coeluted with hydrogenase activity was decreased by about 75% in the peak activity fraction. Of the 1.75 nmol of hydrogenase passed through the column, 67% or 1.17 nmol were inhibited by C_2H_2. The radioactivity in fractions one through four corresponded to 0.58 nmol of $^{14}C_2H_2$. The substoichiometric amount of C_2H_2 probably reflects the release of some bound C_2H_2 from hydrogenase during the time required to process the sample. This is consistent with the experiment described above (Fig. 1) where the sample taken at the first time point already contained a significant amount of C_2H_2. In the experiment described in Fig. 2, the C_2H_2 released during the time (about 20 min) required to process the samples would not have remained in the enzyme solution.

To further investigate the tightness of the binding of C_2H_2 to hydrogenase, samples of the enzyme that had been inhibited with $^{14}C_2H_2$ were treated with SDS-sample buffer, electrophoresed and then fluorographed. The fluorogram revealed two

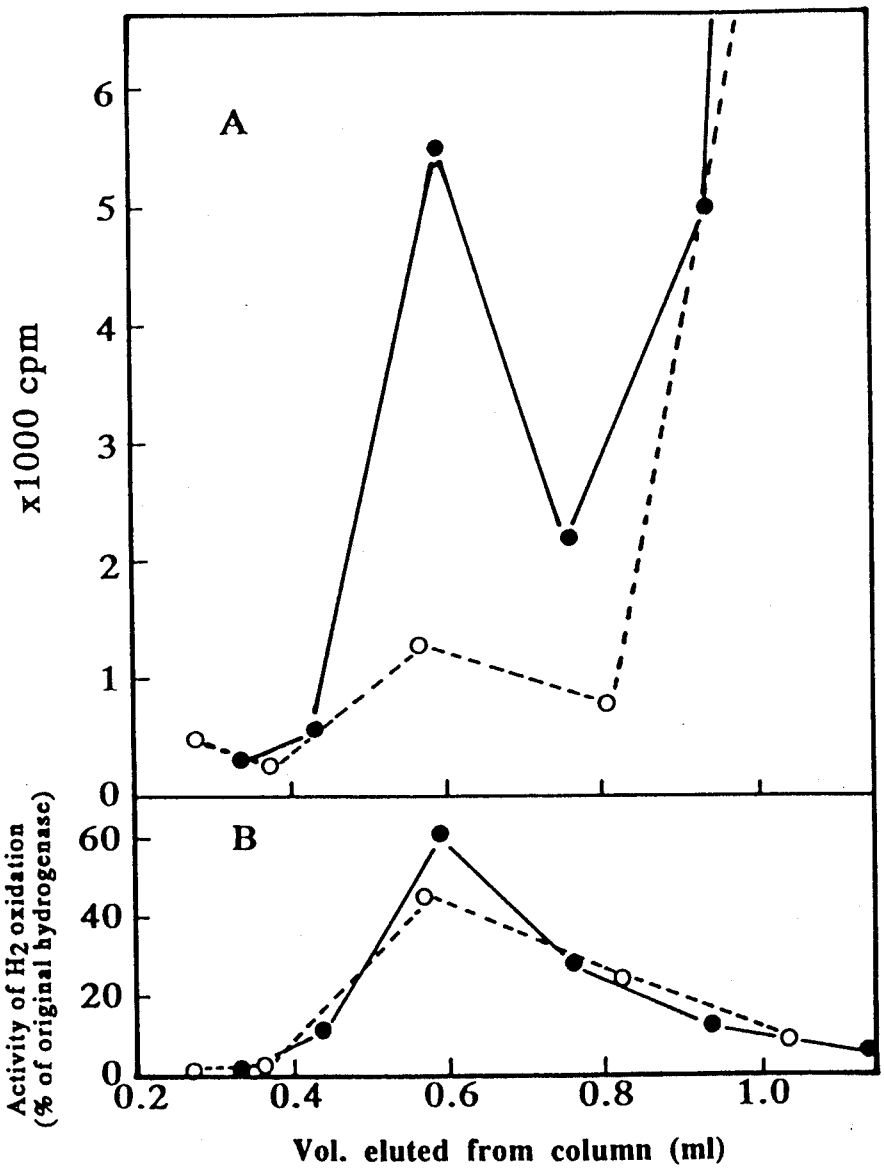


Figure II. 2. Coelution of Radioactivity and Hydrogenase Activity from a Gel Permeation Column Following Inhibition of Hydrogenase with $^{14}C_2H_2$. As described in Materials and Methods, hydrogenase was inhibited with $^{14}C_2H_2$ (•) or $^{14}C_2H_2$ plus H_2 (o) followed by separation of bound and unbound acetylene by passage through a Sephadex G-25 column. Column fractions were analyzed for radioactivity (Panel A) and hydrogenase activity (Panel B).

bands of radioactivity associated with $^{14}C_2H_2$-inhibited hydrogenase (Fig. 3). The bands were greatly diminished in intensity when the hydrogenase was incubated with H_2 and $^{14}C_2H_2$ prior to electrophoresis. Of the two bands of radioactivity revealed in the fluorogram (Fig. 3), the most intense band corresponded with the large subunit of the hydrogenase as indicated by comparison with the gel stained for protein. No radioactive band was detected in the region of the gel corresponding to the small subunit of hydrogenase. Some degradation of the small subunit was apparent (Fig. 3, lane 2) and the extent of degradation increased during the long incubation period whether in the presence (lane 3) or absence (lane 4) of C_2H_2. Note that the degradation did not affect the activity; the control retained complete activity. Thus, of the two hydrogenase subunits, label was associated only with the large subunit.

The weak band of radioactivity revealed in the fluorograms (Fig. 3) corresponded with a very weak protein-staining band which only appeared in the C_2H_2-treated sample (Fig. 3a). The apparent molecular weight of this C_2H_2-induced band was near 90,000. This weak protein-staining band was reminiscent of the weak activity-staining band observed in preparations of *T. roseopersicina* hydrogenase (Kovacs et al., 1991). The origin of this weak band was further investigated in a separate experiment in which *A. vinelandii* hydrogenase was inhibited completely with unlabeled C_2H_2 and then analyzed by SDS-PAGE. The new band was not present prior to C_2H_2 treatment and was not detected in a sample treated with C_2H_2 and H_2 even after an overnight exposure. When the C_2H_2-inhibited sample was allowed to recover activity, the band disappeared indicating that its formation was reversible. The time course of the formation of this band corresponded with the progress of C_2H_2 inhibition (data not shown); the intensity of the band did not continue to increase after C_2H_2 inhibition was complete. Clearly, the formation of this weak band is induced during the inhibition of hydrogenase by C_2H_2 and persists so long as hydrogenases continues to be inhibited by C_2H_2.

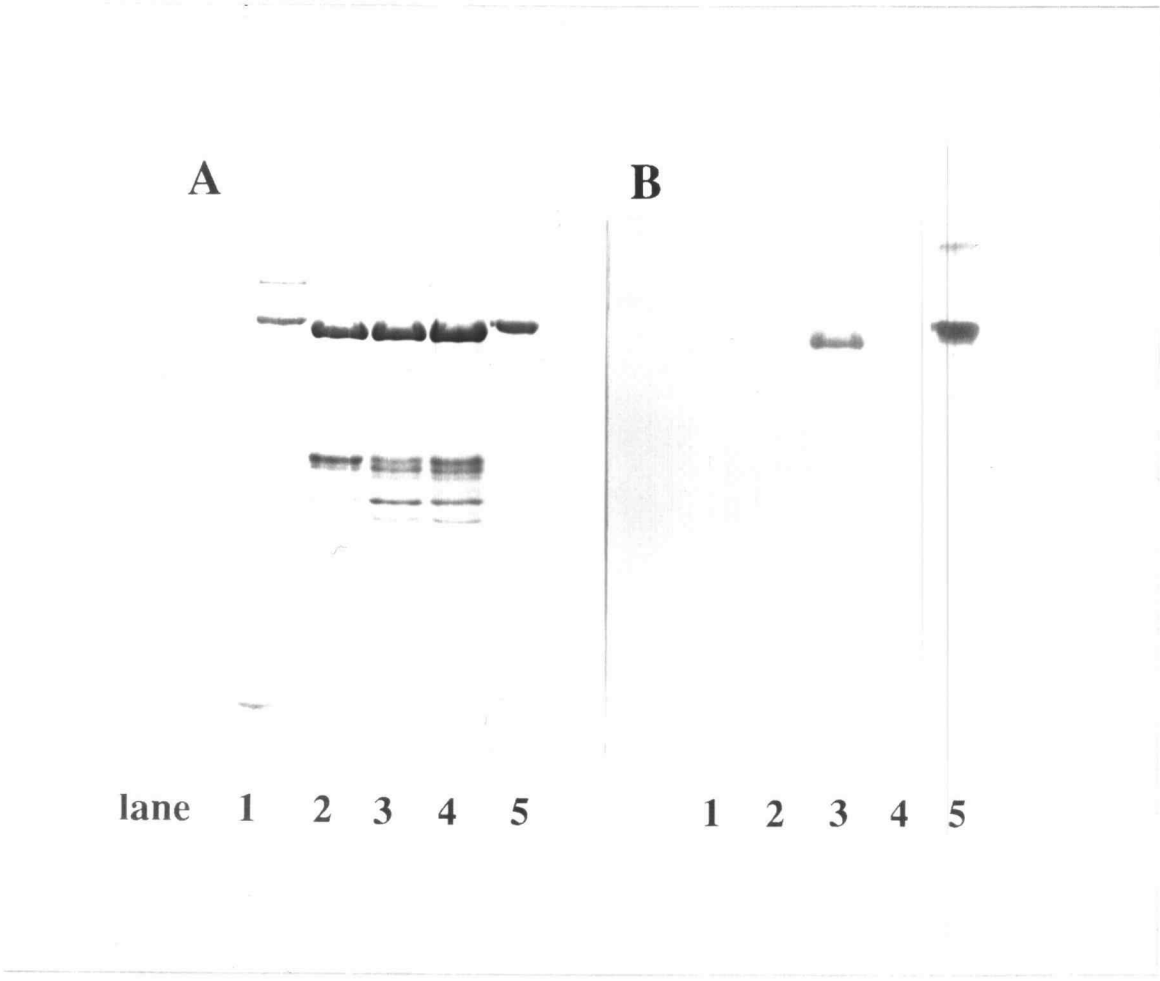


Figure II. 3. SDS-PAGE and Fluorography of $^{14}C_2H_2$-inhibited Hydrogenase. Hydrogenase samples were inhibited with $^{14}C_2H_2$ with or without H_2 as described in the Materials and Methods. Samples (7.1 μg protein) were then analyzed by SDS-PAGE and the gels were stained for protein (Panel A) then prepared for fluorography (Panel B). Lane 1: Molecular weight standards. Lane 2: Uninhibited hydrogenase. Lane 3: $^{14}C_2H_2$-inhibited hydrogenase. Lane 4: Hydrogenase exposed to $^{14}C_2H_2$ plus H_2. Lane 5: ^{14}C-labeled bovine serum albumin (1000 cpm).

In order to conclude that ^{14}C-label was present only on the large subunit and not on the small subunit, it was important to demonstrate that treatment of hydrogenase with C_2H_2 did not alter the ability of the protein to dissociate in the presence of SDS nor did it alter the migration properties of the subunits when electrophoresed in the presence of SDS. Therefore, hydrogenase was inhibited with unlabeled C_2H_2, electrophoresed in the presence of SDS, transferred from the gel to nitrocellulose and then probed with antibodies directed against either the large or small subunit of *B. japonicum* hydrogenase. These immunoblots revealed that the large subunit migrated normally, even when inhibited with C_2H_2, and contained only large subunit; that is, there was no small subunit detected at the position of the large subunit (data not shown). Likewise, the small subunit migrated normally. Therefore, inhibition by C_2H_2 had not altered the dissociation properties of the majority of the hydrogenase. The weak band which formed only when hydrogenase was inhibited with C_2H_2 consisted of the large subunit from hydrogenase as revealed by the immunoblots. Although no small subunit was detected in this weak band, its presence could not be ruled out given the small amount of the new band that formed and the higher detection limit for the small subunit antibody (Kovacs et al., 1989).

Retention of label with a polypeptide following treatment with SDS is often taken as an indication of covalent attachment of the ^{14}C-labeled precursor to the polypeptide. To further probe the chemical basis of this labeling, hydrogenase samples in SDS-PAGE sample buffer were precipitated with TCA (10% w/v), or first heated (95∞C for 10 min) or treated with urea (8 M), prior to precipitation with TCA, then resuspended in SDS sample buffer and electrophoresed and prepared for fluorography. None of these treatments resulted in any detectable loss of label from the protein, confirming that the label is indeed tightly bound to the large subunit.

Acetylene is a time-dependent inhibitor of hydrogenase. Therefore, the time-dependency of the binding of ^{14}C from $^{14}C_2H_2$ to hydrogenase was investigated to

determine if it corresponded to the time course of inhibition. When samples of hydrogenase were analyzed during the time course of an inhibition experiment with $^{14}C_2H_2$, a time-dependent increase in the level of radioactivity on the gel was observed (Fig. 4). For the reasons discussed above, a low concentration of high specific radioactivity acetylene was used in this experiment (about 4 kPa). This limited the extent of inhibition and the resolution of the experiment. Nonetheless, within the limitations of the experiment, a decrease in hydrogenase activity correlated with an increase in radioactivity associated with the large subunit. The level of radioactivity incorporated did not continue to increase when the activity reached a constant value. This is the expected result if the binding of ^{14}C from $^{14}C_2H_2$ and loss of activity are, indeed, related.

^{14}C Is Released from Hydrogenase during Recovery from Inhibition by $^{14}C_2H_2$

The results of Fig. 1 indicated that C_2H_2 was released from hydrogenase during recovery from C_2H_2 inhibition. Therefore, we expected that the ^{14}C bound to hydrogenase should also be released during the recovery from inhibition by $^{14}C_2H_2$. To test this expectation, hydrogenase was inhibited with $^{14}C_2H_2$, then activity was allowed to recover following removal of the unbound $^{14}C_2H_2$. Samples were removed throughout the recovery period and analyzed by SDS-PAGE and fluorography. The ^{14}C attached to the protein during inhibition of hydrogenase with $^{14}C_2H_2$ was released during the recovery period (Fig. 5). The time course of recovery (Fig. 5a) and the amount of label remaining with the protein (Fig. 5b) throughout the recovery period are shown. The label was released from both the large subunit and the weak C_2H_2-induced band.

This experiment also confirmed an important point indicated by the experiment reported in Fig. 1, namely, that the amount of activity recovered and the amount of label

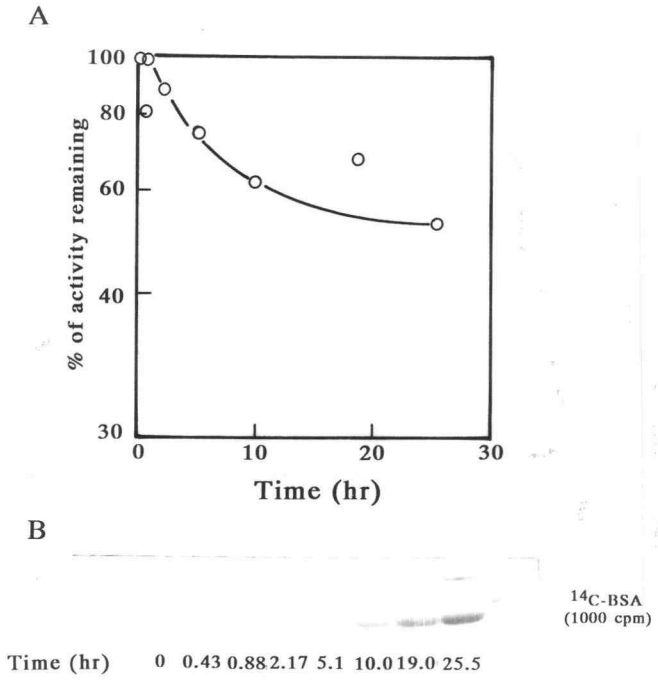
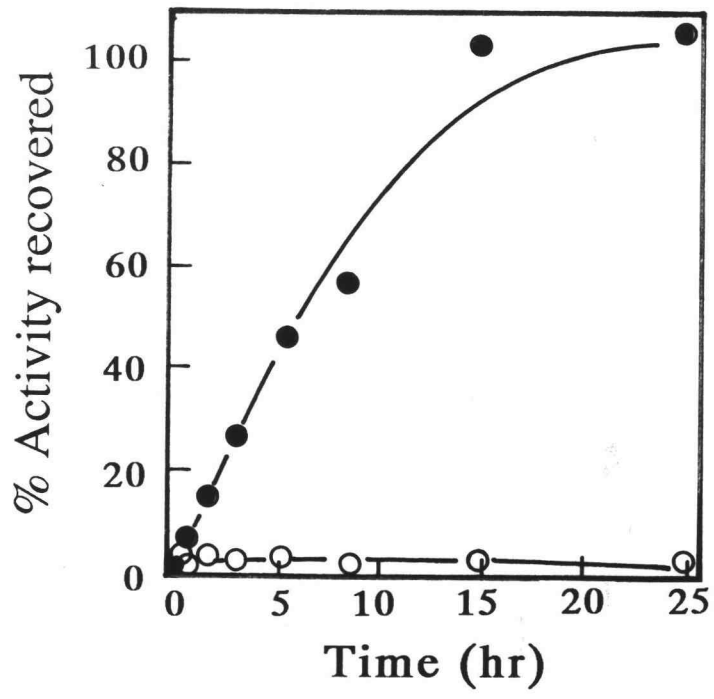
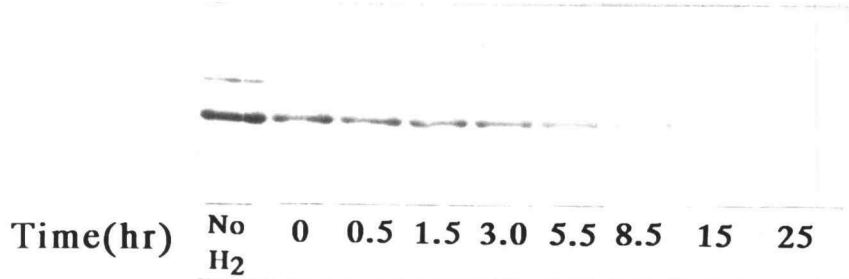


Figure II. 4. Time Course of ^{14}C-labeling and Inhibition of Activity of Hydrogenase by $^{14}C_2H_2$. Purified hydrogenase (1.30 mg/ml) was incubated with 4 kPa $^{14}C_2H_2$ and 97 kPa Ar. At the indicated times, a sample (1 µl) was taken to determine hydrogenase activity (panel A) and another sample (10 µl) was taken and mixed with 50 µl of SDS-PAGE sample buffer for further analysis by SDS-PAGE and fluorography (panel B).

Figure II. 5. Time Course of the Loss of ^{14}C from and Recovery of Activity by Hydrogenase Inhibited with $^{14}C_2H_2$. $^{14}C_2H_2$-inhibited hydrogenase (20 µl,1.5 mg/ml) was mixed with an anaerobic solution of ovalbumin (80 µl, 1 mg/ml; to serve as a carrier protein) in an Eppendorf tube placed in an N_2-filled vial (10 ml). After equilibration of the solution with the gas phase, aliquots of the solution were removed and incubated with 101 kPa H_2 or101 kPa C_2H_2 . At the indicated times, a sample (1 µl) was taken for determination of hydrogenase activity. Panel A: Recovery of hydrogenase activity in samples incubated in H_2 (•) or C_2H_2 (o). A second sample (3 µl) was removed and mixed with 50 µl of SDS-PAGE sample buffer for analysis by SDS-PAGE and fluorography. Panel B: Fluorogram for hydrogenase incubated in H_2. Panel C: Fluorogram for hydrogenase incubated in C_2H_2.


A
% Activity recovered
100
80
60
40
20
0
0
5
10
15
20
25
Time (hr)


B
Time(hr)
No H2
0
0.5
1.5
3.0
5.5
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15
25

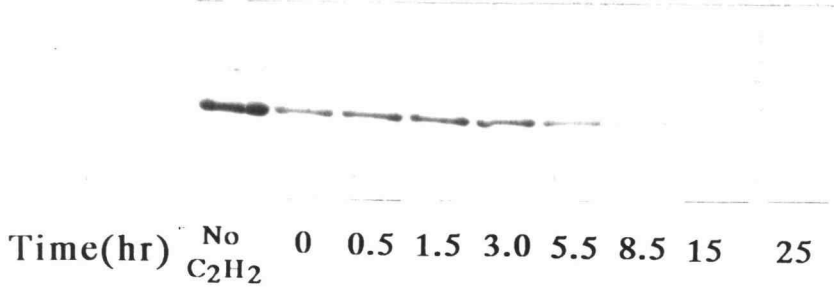
C
Time(hr)
No C2H2
0
0.5
1.5
3.0
5.5
8.5
15
25

Figure II. 5.

lost were not proportional throughout the time course. This was most evident in the first 3 hr of the incubation where only 20% of the activity was recovered but a substantially greater proportion of the radioactivity had been lost. There was also a substantial loss of ^{14}C during the time required to set up the incubation (compare "No H_2" taken at the end of the $^{14}C_2H_2$ inhibition and the 0 hr time point). Another important point revealed by this experiment is that the rate at which label was released from native hydrogenase, although slow relative to that of catalytic turnover, was rapid relative to the rate of release of label from denatured protein. Although label was completely lost from native protein during the 24 hr required for recovery of activity, label remained attached to the denatured protein during the several days required to expose fluorograms.

To further investigate the rate of release of ^{14}C from native hydrogenase, we incubated ^{14}C-labeled protein in the presence of unlabeled C_2H_2 over the same time period required for recovery of activity (Fig. 5). Although the enzyme remained inhibited because of the continued presence of C_2H_2, the amount of label associated with the protein decreased with time (Fig 5c). The time course of the loss of label was virtually identical to that observed when ^{14}C-labeled hydrogenase was incubated in the presence of H_2 and allowed to recover activity.

The Inhibition Is Specific for C_2H_2

The possibility was considered that other compounds might also cause a time-dependent inhibition of hydrogenase activity, similar to the inhibition by C_2H_2. No inhibition, either rapid-equilibrium or time-dependent, was observed when hydrogenase was incubated with 101 kPa of either ethylene, ethane or methane. Furthermore, no time-dependent inhibition was observed when hydrogenase was incubated with the hydrolysis product of acetylene, acetaldehyde (1 mM), or the oxidation products of acetylene, ethanol (40 mM), acetate (1 mM), or glyoxylate (1 mM).

For some metalloenzymes for which C_2H_2 is an inhibitor, e.g. nitrogenase and ammonia monooxygenase, other alkynes in addition to C_2H_2 are inhibitors (Hyman and Arp, 1988). To explore this possibility with hydrogenase, the enzyme was incubated for 60 min with 101 kPa propyne or 1-butyne. The solution concentrations of propyne (81.2 mM) and 1-butyne (72.9 mM) were high relative to the solution concentrations of C_2H_2 required for inhibition over this time period. Nonetheless, no inhibition of hydrogenase activity was observed in the presence of propyne. Some inhibition was observed when hydrogenase was treated with 1-butyne (37 % loss of activity after 60 min), but the level of inhibition was consistent with the small amount of C_2H_2 (1.7 kPa) which contaminated the 1-butyne. When C_2H_2 (50 kPa) was added to the vials, inhibition proceeded normally. This indicated that the presence of propyne or 1-butyne did not prevent the binding of C_2H_2. These results, taken together with the results described above, indicate that the inhibition by C_2H_2 is remarkably specific for C_2H_2.

Acetylene as an Analogue of H_2

As discussed below, several lines of evidence support the idea that C_2H_2 acts as an analogue of H_2. To further pursue this concept, two additional experiments were carried out. A small kinetic isotope effect is observed for related hydrogenases when D_2 is the substrate for hydrogenase instead of H_2 (Arp and Burris, 1981). To determine if there is an observable kinetic isotope effect on the rate of acetylene inhibition, both C_2H_2 and C_2D_2 were prepared and used to inhibit hydrogenase. Gas chromatography was used to verify that the same concentration of acetylene was present in each case. The liquid phase in these reaction mixtures contained H_2O and C_2D_2 would be expected to exchange with solvent protons to form C_2HD and C_2H_2. Therefore, the isotopic composition of the acetylene was determined by mass spectrometry and the exchange reaction was found to be slow (about 10% of the C_2D_2 exchanged in 24 hr) relative to the

rates of inhibition at the pH used in the experiment. When hydrogenase was exposed to either C_2D_2 or C_2H_2, the rate of inhibition was identical. This indicates that the rate-limiting step in the inhibition is not influenced by the isotopic composition of the C-H bond in acetylene.

H_2 protects hydrogenase from irreversible inactivation by O_2 (Seefeldt and Arp, 1989b). If C_2H_2 and H_2 bind analogously to hydrogenase, then perhaps C_2H_2 could also protect hydrogenase from irreversible inactivation by O_2. To test this possibility, hydrogenase was first inhibited with C_2H_2 (101 kPa for 4 hr resulting in 100% inhibition of activity). The gas phase was then changed to air (101 kPa) and the enzyme was incubated for an additional 24 hr. This length of exposure to air was sufficient for complete inactivation of a sample not pretreated with C_2H_2 (Seefeldt and Arp, 1989b). The air was then evacuated and replaced with H_2 (101 kPa) and the enzyme was incubated for an additional 52 hr (the time required for recovery from C_2H_2 inhibition). During this incubation, hydrogenase activity was recovered (99-103% of the original activity). This result indicates that C_2H_2, like H_2, can protect hydrogenase from irreversible inactivation by O_2.

Discussion

Acetylene inhibits a number of metalloenzymes, including nitrogenase, ammonia and methane monooxygenases, nitrous oxide reductase and hydrogenase (Hyman and Arp, 1988). The mechanism of the inhibition varies with the enzyme. For example, C_2H_2 is an alternative substrate for nitrogenase which inhibits N_2 reduction by competing for reductant and ATP. With ammonia and methane monooxygenases, C_2H_2 is a mechanism-based inactivator. The catalytic activity of the monooxygenases activates C_2H_2 to a reactive intermediate which binds irreversibly to the enzyme. For hydrogenases, C_2H_2 was described as an active-site directed, slow-binding inhibitor (Hyman and Arp, 1987a). The slow binding of C_2H_2 to hydrogenase results in a time-dependency of the inhibition. The inhibition is reversible, albeit slowly, when the C_2H_2 is removed. The following observations have led to the idea that C_2H_2 acts as an analogue of H_2. 1) H_2 protects hydrogenase from inhibition by C_2H_2 and the interaction of H_2 and C_2H_2 with hydrogenase is competitive (Hyman and Arp, 1987a). 2) Both H_2 activation and C_2H_2 inhibition require catalytically competent enzyme (Hyman et al., 1988). 3) Neither H_2 nor C_2H_2 alters the EPR spectrum associated with dithionite-reduced hydrogenase and both H_2 and C_2H_2 cause a similar change in the EPR spectrum of O_2-inhibited hydrogenase (Seefeldt, L.C., Ph.D. Thesis, 1989). 4) Both H_2 (Seefeldt and Arp, 1989b) and C_2H_2 (this work) protect hydrogenase from irreversible inactivation by O_2. In contrast, CO (another hydrogenase inhibitor which is competitive vs. H_2) does not protect hydrogenase from irreversible inactivation by O_2 (Seefeldt and Arp, 1989b). Given these similarities, we considered the possibility that C_2H_2 was transformed by hydrogenase to another compound, i.e. that C_2H_2 acted as a substrate for hydrogenase. However, the fact that C_2H_2 is released from hydrogenase in amounts nearly stoichiometric with hydrogenase (Fig. 1), and our failure to detect other putative products, indicates that C_2H_2 is not transformed to another compound either as a

mechanism leading to inhibition of hydrogenase or as a mechanism of recovery from inhibition.

Acetylene Binds reversibly to *A. vinelandii* Hydrogenase

The results of this work (Fig. 1,2,3) clearly demonstrate that C_2H_2 (or a derivative of C_2H_2) does, indeed, bind tightly to *A. vinelandii* hydrogenase. Although precise quantitation is difficult, the analysis of the data from Fig. 1 and 2 support a 1:1 stoichiometry of C_2H_2 bound to hydrogenase. We had previously shown that purified hydrogenase could at least partially recover activity when C_2H_2 was removed (Hyman and Arp, 1987a). In this work, we demonstrate that the recovery can be complete (e.g., Fig. 1,5), but requires from 15 to 70 hr to recover fully. The reason for the variability of recovery times is not known. During the recovery of activity from C_2H_2 inhibition, C_2H_2 was released from the native enzyme (Fig. 1). However, the release of C_2H_2 and the recovery of activity were not coincident (Fig. 1,5). Acetylene was released more rapidly than activity was recovered. This result was demonstrated by two independent techniques, namely, measurement by gas chromatography of the C_2H_2 released during the time course of recovery (Fig. 1) and determination of the relative amount of ^{14}C-label associated with hydrogenase during the recovery (Fig. 5). Apparently, C_2H_2 release from the enzyme is a requirement for, but not in itself sufficient for, recovery of activity. This suggests that there are three forms of the hydrogenase present during the recovery period. The first is inhibited hydrogenase with C_2H_2 or an C_2H_2-derived adduct attached ([EI]'), the second is inactive hydrogenase with no C_2H_2 attached (E'), and the third is active hydrogenase (E). Thus, a two-step recovery of activity is indicated as illustrated below where k_5 is the rate constant for conversion of [EI]' to E' and k_7 is the rate constant for the conversion of E' to E.

$$E + I \underset{k2}{\overset{k1}{<====>}} EI \underset{k4}{\overset{k3}{<====>}} [EI]' \underset{k6}{\overset{k5}{<====>}} E' + I \underset{k8}{\overset{k7}{<====>}} E + I$$

{---Inhibition---} {---Recovery---}

The inhibition phase (formation of [EI]') was discussed previously (Hyman and Arp, 1987a) and none of the experiments reported here provide any additional insight into the kinetic mechanism of C_2H_2 inhibition. We favor the mechanism depicted above which implies a saturable rate of tight, but reversible, complex formation (Schloss, 1988). However, given the relatively weak inhibition, a simpler mechanism in which the tight, reversible complex, [EI]', is formed directly cannot be ruled out. The release of C_2H_2 prior to recovery of activity would suggest that [EI]' is not converted back to EI and E directly, i.e. k_2 and k_4 are very slow. Rather, the [EI]' must first proceed to E' (at rate k_5) which then slowly converts to E at rate k_7. ^{14}C-label was released from the protein with the same kinetics in the presence or absence of unlabeled C_2H_2 (Fig. 5) which is consistent with this model. While the continued presence of C_2H_2 prevents recovery of activity, this experiment does not reveal if this occurs by direct binding of C_2H_2 to E' or follows the reaction sequence

E' > E > EI > [EI]'.

^{14}C-Label from $^{14}C_2H_2$ Is Bound to the Large Subunit of *A. vinelandii* Hydrogenase

Analysis by SDS-PAGE and fluorography of $^{14}C_2H_2$-inhibited hydrogenase revealed that label was associated with the large subunit (Fig. 3). This result was surprising given the reversible nature of the inhibition and binding of C_2H_2 to native protein. Clearly, the label is bound more stably to SDS-denatured protein than to the

native protein. Furthermore, none of the additional denaturing treatments resulted in the release of the label. Apparently, denaturation "locks" the C_2H_2-derived label onto the protein, perhaps through a covalent interaction of the C_2H_2 with hydrogenase. The mechanism of inhibition of hydrogenase by C_2H_2 may involve the covalent attachment of C_2H_2 to the protein and denaturation simply eliminates the possibility of a back reaction by disruption of the active site. For example, if Ni or an FeS cluster are required for inhibition and for reversibility, then their removal by denaturation would eliminate the possibility of a back reaction.

Label from $^{14}C_2H_2$ binds to the large subunit and not the small subunit as demonstrated by the correspondence of the radioactive band with the large subunit through protein (Fig. 3) and immunostaining (not shown). The attachment of label from $^{14}C_2H_2$ to the large subunit leads to an important finding regarding the role of the large subunit in catalysis. Given that C_2H_2 behaves as an analogue of H_2 and that label from $^{14}C_2H_2$ is attached only to the large subunit, it follows that the large subunit most likely contains the site of H_2 activation. As such, our experiments provide the first biochemical evidence that the H_2-activating site is located on the large subunit. This idea is consistent with other observations as discussed in a recent review (Przybyla et al., 1991). Our experiments also provide the first description of an active-site directed inhibitor of hydrogenase activity that binds sufficiently tightly to remain bound following denaturation of the protein. Such an inhibitor should be useful in further delineating the active site of hydrogenase.

We can speculate on a model for the mechanism of the binding of C_2H_2 to hydrogenase which is consistent with the experimental results. To obtain the apparently covalent attachment of C_2H_2 to hydrogenase, C_2H_2 must be activated by the enzyme. Given that C_2H_2 behaves as an analogue of H_2, the activation of C_2H_2 should bear some resemblance to the activation of H_2. In the oxidation of H_2, a heterolytic split of H_2 is

proposed, resulting in formation of a Ni-hydride species and a proton bound to a base (Przybyla et al., 1991). In the inhibition of hydrogenase by C_2H_2, the relatively acidic proton of C_2H_2 could be abstracted upon binding to Ni, resulting in formation of Ni acetylide. The acetylide, which is a strong base, could then react with R groups in the active site to form the stable attachment of an acetylene-derived carbon to protein. As discussed above, this may occur only upon denaturation of the protein, or it may be that the covalent attachment is a part of the inhibition mechanism and that denaturation eliminates the pathway for the back reaction. In either event, it is clear that the reaction must be reversible in the native protein.

Summary

Through investigation of the mechanism of C_2H_2 binding to hydrogenase, we have demonstrated the following: 1) C_2H_2 binds tightly and reversibly to native hydrogenase. 2) Hydrogenase does not catalyze the transformation of C_2H_2 to another compound. 3) The inhibition is remarkably specific for C_2H_2. 4) Inhibition of hydrogenase by C_2H_2 results in the formation of a new protein-staining band of weak intensity which binds C_2H_2. 5) Denaturation of hydrogenase inhibited with $^{14}C_2H_2$ reveals the binding of ^{14}C to the large subunit of hydrogenase which provides the first biochemical evidence that the H_2-activating site of a NiFe dimeric hydrogenase is located on the large subunit.

References

Adams, M. W. W., Eccleston, E. & Howard, J. B. (1989) *Proc. Natl. Acad Sci. USA 86*, 4932-4936.

Arp, D. J. & Burris, R. H. (1981) *Biochemistry 20*, 2234-2240.

Bagyinka, C., Szokefalvi-Nagy, Z., Demeter, I. & Kovacs, K. L. (1989) *Biochem. Biophys. Res. Commun. 162*, 422-426.

Birkett, C. R., Foster, K. E., Johnson, L. & Gull, K. (1985) *FEBS Lett. 187*, 211-218.

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83-88.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 245-254.

Eidsness, M. K., Scott, R. A., Prickril, B., DerVartanian, D. V., LeGall, J., Moura, I., Moura, J. J. G. & Peck, H. D. (1989) *Proc. Natl. Acad. Sci (U.S.A.) 86*, 147-151.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem. 177*, 751-766.

Hathaway, G. M., Lundak, T. S., Tahara, S. M. & Traugh, J. A. (1979) *Methods Enzymol. 60*, 495-511.

He, S. H., Teixeira, M., LeGal, J., Patil, D. S., Moura, I., Moura, J. J. G., DerVartanian, D. V., Huynh, B. H. & Peck, H. D. (1989b) *J. Biol. Chem. 264*, 2678-2682.

He, S. H., Woo, S. B., DerVartanian, D. V., LeGall, J. & Peck, H. D. (1989a) *Biochem. Biophys. Res. Commun. 161*, 127-133.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1990) *Anal. Biochem. 190*, 348-353.

Hyman, M. R. & Arp, D. J. (1988) *Anal. Biochem. 173*, 207-220.

Hyman, M. R. & Arp, D. J. (1987a) *Biochemistry 26*, 6447-6454.

Hyman, M. R. & Arp, D. J. (1987b) *Appl. Environ. Microbiol. 53*, 298-303.

Hyman, M. R., Seefeldt, L. C. & Arp, D. J. (1988) *Biochim. Biophys. Acta 957*, 91-96.

Juszczak, A., Aono, S. & Adams, M. W. W. (1991) *J. of Biol. Chem. 266*, 13834-13841.

Kovacs, K. L., Seefeldt, L. C., Tigyi, G., Doyle, C. M., Mortenson, L. E. & Arp, D. J. (1989) *J. Bacteriol. 171*, 430-435.

Kovacs, K. L., Tigyi, G., Thanh, L., Lakatos, S., Kiss, Z. & Bagyinka, C. (1991) *J. Biol. Chem. 266*, 947-951.

Kow, Y. W. & Burris, R. H. (1984) *J. Bacteriol. 159*, 564-569.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1991) *FEMS Microbiol. Rev. In press.*

Schloss, J. V. (1988) *Accounts Chem. Res. 121*, 348-353.

Seefeldt, L. C. & Arp, D. J. (1989a) *J. Bacteriol. 171*, 3298-3303.

Seefeldt, L. C. & Arp, D. J. (1989b) *Biochemistry 28*, 1588-1596.

Seefeldt, L. C. & Arp, D. J. (1987) *J. Biol. Chem. 262*, 16816-16821.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Smith, L. A., Hill, S. & Yates, M. G. (1976) *Nature 262*, 209-210.

Sun, J. H. & Arp, D. J. (1991) *Arch. Biochem. Biophys. 287*, 225-233.

van der Werf, A. N. & Yates, M. G. (1978) in *Hydrogenases: Their catalytic activity, structure and function* (H. G. Schlegel and K. Schneider, Ed.) pp 307-326, Erich Goltze KG, Goettingen.

Voordouw, G., Menon, N. K., LeGall, J., Choi, E. S., Peck, H. D. & Przybyla, A. E. (1989) *J. Bacteriol. 171*, 1969-1977.

CHAPTER III

UV-VIS ABSORPTION SPECTRA OF DIFFERENT ACTIVE STATES OF *AZOTOBACTER VINELANDII* HYDROGENASE TREATED WITH O_2, C_2H_2, CN^- AND NO

by

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Running title: Absorption spectrum of hydrogenase

Contributions

Jin-hua Sun participated in developing the experimental design and the techniques utilized to perform this study, performing the experiments, analyzing the data and calculating the results. Dr. Daniel J. Arp participated in the experimental design, supervising pregress of study and editing the manuscript.

Abbreviations

EPR; electron paramagnetic resonance

MES; 2-(N-morpholino)ethanesulfonic acid

SDS; sodium dodecylsulfate

SDS-PAGE; sodium dodecylsulfate-polyacrylamide gel electrophoresis

Tris; tris(hydroxymethyl)aminomethane

UV-vis.; ultraviolet-visible

Abstract

Hydrogenase from *Azotobacter vinelandii* exists in variety of active states upon incubation with O_2, C_2H_2, CN^-, or NO. UV-visible absorption spectra of these active states are very different. The isolated, active state of enzyme with N_2 or H_2 exhibited a considerable absorbance in the 530-300 nm range. Upon incubation with O_2 in the presence of H_2, hydrogenase was reversibly inhibited, and showed a broad absorption band from 530 to 380 nm and a small absorption shoulder from 380 to 300 nm. These two bands were centered at 435 nm and 345 nm respectively in difference spectra of O_2-inhibited enzyme *minus* the active enzyme and these differences decreased with recovery of activity by incubation with dithionite and H_2. Long term incubation with O_2 resulted in an irreversibly inactivated state. In this state, the enzyme showed a ΔA peak at 315 nm, in addition to the ΔA435nm and ΔA345nm peaks. As in the O_2 inhibited enzyme, ΔA435nm and ΔA345nm peaks were partially reduced upon incubation with H_2. But the ΔA315nm peak could not be reduced. Compared with isolated, active hydrogenase, the C_2H_2-inhibited enzyme was less sensitive to the irreversible O_2 inactivation, but susceptible to reversible O_2 inhibition. In the absorption spectra, the C_2H_2-inhibited hydrogenase showed Δ A peaks at 491.5 nm, 338 nm, and 289 nm. The C_2H_2-inhibited enzyme was able to exhibit the ΔA435nm peak if O_2 was present. CN^- inactivated hydrogenase showed no tritium exchange activity, suggesting CN^- reacted with H_2 activation site. In the absorption difference spectra, ΔA310 peak, ΔA340nm shoulder and ΔA380nm trough were observed. These absorption changes are O_2-dependent as is the inactivation of enzyme. The broad absorption band from 530 to 380 nm which resulted from the treatment with O_2 during CN^- inactivation could not be reversed by incubation with H_2, but could be reversed by treatment with dithionite. NO treatment resulted in irreversible inactivation of hydrogenase activity. In absorption spectra, NO, besides its absorption at 350 nm, induced the broad absorption band in the range of 530 -300 nm and

especially the increase of absorption at 320 nm. No further effects of O_2 on absorption of the NO-treated enzyme were observed in the range of 530-380 nm.

Introduction

Hydrogenases (EC classes 1.12 and 1.18) are a rather heterogeneous group of metalloenzymes which catalyze the consumption or evolution of the simplest molecule H_2. All hydrogenases appear to contain iron as part of iron-sulfur centers, with exception of methylenetetrahydromethanopterin dehydrogenase (Zirngibl, et al., 1992), although the numbers and types of centers vary with the particular hydrogenase (Adams, 1990). Many hydrogenases have also been shown to contain nickel (Cammack, et al., 1988, Eisbrenner and Evans, 1983), that has been proposed to play an essential role in the activation of H_2, perhaps through formation of a nickel hydride. A few hydrogenases contain selenium which, as a selenocysteinyl ligand, replaced one of the cysteinyl ligands to the nickel (Eidsness, et al., 1989, He, et al., 1989a). The hydrogenase from *Azotobacter vinelandii*, that is expressed under N_2-fixing conditions, is a typical Ni- and Fe-containing enzyme. From comparisons in the amino acid sequence (Menon, et al., 1990), the catalytic properties (Seefeldt and Arp, 1986), contents of prosthetic group(Seefeldt and Arp, 1986), and EPR spectroscopy (Seefeldt, 1989), *A. vinelandii* hydrogenase is similar to the hydrogenases isolated from both N_2-fixing and other microorganisms including *Alcaligenes eutrophus* H16 (particulate), *Bradyrhizobium japonicum*, *Rhodobacter capsulata*, and *Escherichia coli* (hydrogenase-1). However, *A. vinelandii* hydrogenase is less similar to the hydrogenase isolated from sulfate reducing bacteria, such as *Desulfovibrio gigas* and *Desulfovibrio vulgaris*.

The purified *A. vinelandii* hydrogenase is a dimer with subunits of 65 kDa and 31 kDa. Elemental analysis reported 6.6 Fe and 0.7 Ni per molecular (Seefeldt and Arp, 1986). Recently, by analysis of composition of amino acids of the enzyme, it was found that protein concentration was over-estimated by Biuret Method by 1.91±0.31 fold (Sun, et al., 1992). This means that the *A. vinelandii* hydrogenase would contain 12.6 Fe and 1.3 Ni per molecular. Therefore, the possible [Fe-S] cluster inventory for *A. vinelandii*

hydrogenase is at least two [4Fe-4S] and one [3Fe-4S]. The EPR investigation in the *A. vinelandii* hydrogenase revealed signals typical of a [4Fe-4S] cluster interacting with another paramagnet in the reduced enzyme and a g=2.01 signal typical of a [3Fe-4S] cluster in the oxidized state. Given the current knowledge about the Ni-containing hydrogenase, the Ni is not complexed to an organic cofactor, but is bound to the protein through coordination with amino acid residues. The ligands which bind Ni to *A. vinelandii* hydrogenase have not been determined; however, sequence identity in conserved stretches of the gene coding for the large subunit of *A. vinelandii* and other Ni-containing hydrogenases would suggest that the putative ligands include cysteinyl, histidinyl, aspartyl or arginyl residues (Przybyla, et al., 1992).

The interactions of a number of inhibitors with hydrogenase from *A. vinelandii* have been characterized kinetically. CO and C_2H_2 are both competitive vs. H_2, indicating that their binding is mutually exclusive and that each might bind to the H_2 activating site. However, binding of CO follows rapid equilibrium kinetics while the binding and release of C_2H_2 occurs very slowly relative to the time scale of catalytic turnover (Hyman and Arp, 1987a). O_2 (Seefeldt and Arp, 1989b) and NO (Hyman and Arp, 1991) are unique in that they exhibit both reversible and irreversible components to their interaction with hydrogenase. H_2 does not prevent the binding of either of these inhibitors, indicating that each binds at a site other than the H_2-activating site. Although H_2 cannot prevent the binding of O_2, it does prevent the irreversible loss of activity associated with prolonged exposure to O_2. In contrast, H_2 does not prevent the irreversible effects of NO on hydrogenase. The interaction of NO with membrane-bound hydrogenase appears to be even more complex than the interaction of O_2 with hydrogenase, probably owing to the fact that NO can bind to and react differently with each of the [Fe-S] clusters. Cyanide (Seefeldt and Arp, 1989a) does not inhibit the reduced form of the *A. vinelandii* hydrogenase. However, cyanide does irreversibly inactivate the reversibly inactive form of hydrogenase which is produced when

hydrogenase is incubated simultaneously with H_2 and O_2. These inhibitors provide a collection of potential ligands to the redox centers of *A. vinelandii* hydrogenase with which to probe the function of each redox center.

In the present work, we have examined the effects of inhibitor: O_2, C_2H_2, CN^-, and NO on UV-visible spectra of *A. vinelandii* hydrogenase. For providing the basis of interpretation of the effects, if any, on the spectra, we determined the catalytic state of the enzyme before and after treatment with each inhibitor.

Materials and Methods

Materials

H_2, and N_2 (>99.99% purity) purchased form Liquid carbonic Corp (Chicago, IL) were stripped of residual O_2 by passage over a heated copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plaensfield, NJ). Gas from an acetylene cylinder (99.6%) was further purified according to Hyman and Arp (Hyman and Arp, 1987b). All other chemicals were of reagent grade.

Hydrogenase Purification

Cells of *Azotobacter vinelandii* (strain OP) were grown under N_2-fixing conditions as described (Seefeldt and Arp, 1989b). Hydrogenase was purified in the active form under anaerobic and reducing (2 mM dithionite) conditions as described (Sun and Arp, 1991).

The fractions of hydrogenase from Octyl-Sepharose column with a specific activity higher than 100 units/mg were loaded onto a gel filtration column (Sephacryl S-300 1x45 cm). The hydrogenase was eluted by the 0.05 M Tris-HCl (pH 7.4) containing 2 mM dithionite. Hydrogenase was eluted from the column at an elution volume of 40 ml. Gel filtration removed a contaminating protein (MW: 27,600) from the hydrogenase protein. The contaminating protein had an absorption peak at 408 nm in the UV-visible spectrum.

H_2 Oxidation Assays

H_2 oxidation/electron acceptor reduction activity was determined spectrophotometrically at 30 °C in 50 mM MES buffer, pH 6.0, including 0.2 mM methylene blue. All assays were performed in stoppered glass cuvettes with solutions made anaerobic by purging with H_2 gas. The extinction coefficients for the electron acceptors were taken as 11.4 $mM^{-1}.cm^{-1}$ at 690 nm for methylene blue.

Incubation Procedures

Incubations were carried out in stoppered serum vials (13.8 ml) that contained an inner, open-topped reaction chamber (1 ml) (double chamber vial). The required gas phase was added to these stoppered vials after they had been evacuated for 5 min on a vacuum manifold. The final gas pressure was 101 kPa at 25 °C. The enzyme (for absorption spectra) or N_2-purged buffer (20 mM MES pH 6.0, unless stated otherwise) was added to the inner chamber after the vials had been allowed to equilibrate for 15 min with shaking prior to addition of enzyme or buffer. Unless stated otherwise, the incubation or reactions of enzyme with inhibitor were initiated by addition of purified hydrogenase. If the experiment required anaerobic conditions, the vial also contained 0.5 mL of 0.1 M sodium dithionite in buffer in the outer section of the vial to serve as an O_2 scavenger. Prior to the initiation of this kind of experiment, anaerobic buffer was added to the inner vial to allow pre-equilibration with the gas phase for at least 30 min. This step was taken to minimize any limitation on gas diffusion when enzyme was added to the incubation vial. It was confirmed that all of the dithionite carried with the enzyme was consumed by the O_2 within 15 s after addition of sample to the incubation vial (Seefeldt and Arp, 1989b). At the indicated times, an aliquot was removed from the vial and either injected into an assay cuvette or placed into a separate, stoppered vial and

repeatedly evacuated and flushed with whatever required gas was for further experiments. Reported H_2 oxidation rates were the maximum rates achieved during the course of the assay (unless stated otherwise).

UV-vis. Absorption Measurements

Spectra were recorded on a Beckman DU-7 spectrophotometer. Spectra of oxidized and reduced protein samples were recorded from 200 to 700 nm. Protein was stripped of dithionite by passage through a 1x10 cm column of Sephadex G-25 under H_2 or N_2 gas. All column buffers were pre-evacuated/flushed, and then sparged with H_2 or N_2 for 30 min. 50 mM Tris-HCl buffer, pH 7.4, was used in all steps (unless stated otherwise). The protein samples were transferred via gas-tight syringes to stoppered anaerobic quartz cuvettes containing 101 kPa H_2 or N_2. The treatment of hydrogenase by O_2 or other inhibitors were carried out in a double chamber vial (see incubation procedures).

Results

UV-vis. Absorption Spectra of Reduced *A. vinelandii* Hydrogenase

The UV-visible absorption spectrum of *A. vinelandii* hydrogenase reveals a broad absorption envelope in the 300-600 nm region (Fig. III-1) which is typical of iron sulfur centers in proteins (Sweeney, 1980). The millimolar extinction coefficients at 279 and 435 nm are 162.7 $cm^{-1}.mM^{-1}$ and 16.4 $cm^{-1}.mM^{-1}$ respectively. Although hydrogenase was purified in the presence of dithionite (which absorbs long wavelength UV light), this reductant was removed by gel permeation chromatography prior to recording the spectrum. When H_2 was added to the hydrogenase sample, no change in light absorption was observed. H_2 binds to and is activated by this form of the enzyme, as demonstrated by the isotope exchange reaction (Seefeldt, et al., 1986); however, the binding of H_2 does not cause any changes that are reflected in the absorption properties of the chromophores giving rise to the UV-vis spectrum.

The final step in the purification (gel permeation chromatography) removes a protein which also absorbs visible light. The absorption spectrum of this protein was typical of a cytochrome. The major absorption peak of the protein as isolated under reducing conditions was present at 408 nm but the peak shifted to 415.5 nm upon exposure to O_2. There is an open reading frame (ORF) immediately downstream from the two ORF's that code for the small and large subunits of *A. vinelandii* hydrogenase (Menon, et al., 1990). Analysis of the sequence of the third ORF suggests that the putative protein product of this ORF could be a membrane protein. Therefore, we considered the possibility that the protein we had isolated and which co-purified with hydrogenase up to the final step, was in fact coded for by ORF 3 in the hydrogenase gene. To check this possibility, the N-terminal sequence of the protein was determined.

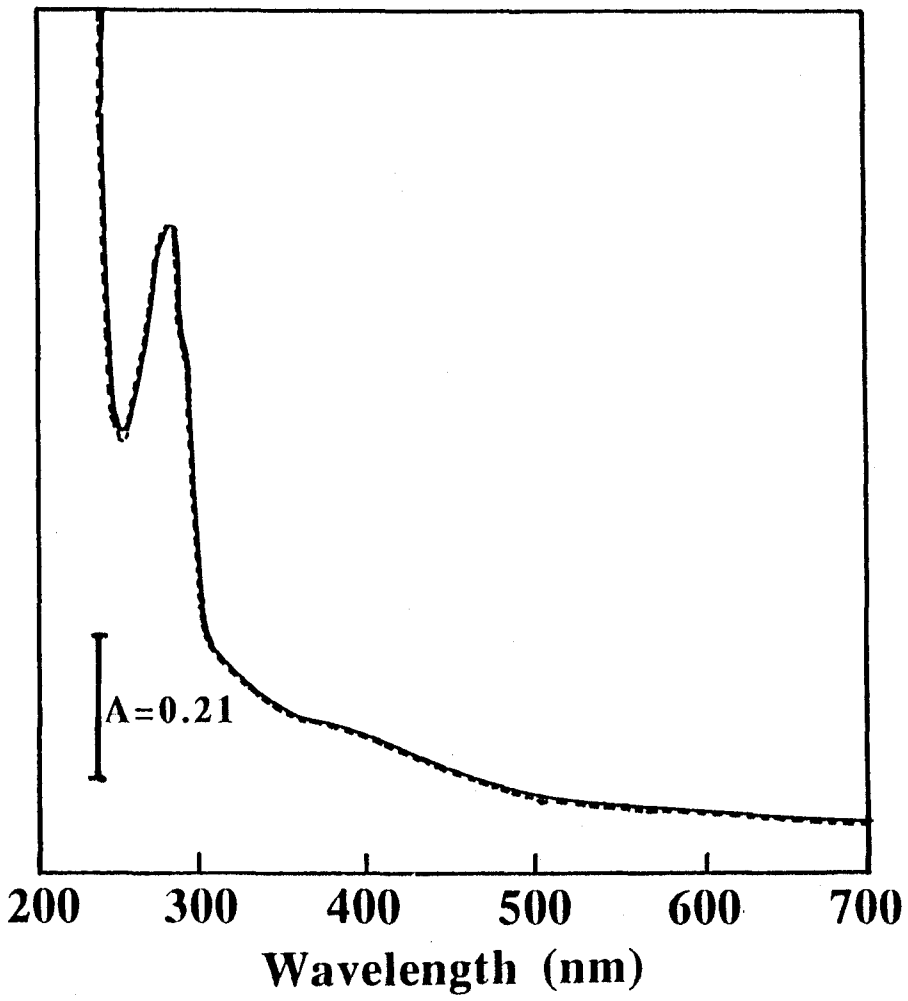

Figure III. 1. UV-vis. Absorption Spectra of *A. vinelandii* Hydrogenase under N_2 or H_2 Gas-phase. The isolated hydrogenase was stripped of dithionite under N_2. 1 ml of enzyme (0.54 mg protein. ml^{-1}) was scanned in a N_2-filled cuvette (——), then the enzyme was evacuated and equilibrated with H_2 for 60 min.and a new spectrum was recorded in the H_2-filled cuvette (-----).

However, the sequence did not match any portion of the predicted sequence of the putative protein product of ORF 3 and, therefore, cannot be the product of this ORF.

UV-vis. Absorption Spectra of O_2-Oxidized Forms of *A. vinelandii* Hydrogenase

When *A. vinelandii* hydrogenase is exposed simultaneously to H_2 and O_2, the enzyme is inactive, but the activity can be recovered by removal of the O_2 (Seefeldt and Arp, 1989b). Upon treatment of hydrogenase with H_2 and O_2, the absorption of UV and visible light revealed a broad absorption band from 530 to 380 nm and an absorption shoulder from 380 to 300 nm relative to enzyme exposed to H_2 only (Fig. III-2A). The difference spectrum for hydrogenase exposed to H_2 and O_2 *minus* hydrogenase exposed to H_2 showed peaks at about 350 and 430 nm (Fig. III-2A, Inset). Similar absorption maxima were also observed when hydrogenase was incubated with H_2 and O_2 at pH 5.0 (1 mM NH_4HCO_3) and pH 9.0 (50 mM glycine) (data not shown), indicating that the absorption changes occurred over a wide pH range. Peaks in this region are characteristic of the oxidation of [Fe-S] clusters (Sweeney, 1980). The increases in absorption were largely reversed when the O_2 was removed from hydrogenase (Fig. III-2A). Thus, the reversible changes in the UV-visible absorption spectrum upon exposure to H_2 and O_2 were consistent with the reversible oxidation of [Fe-S] cluster(s) and with the reversible inhibition of enzyme activity.

When *A. vinelandii* hydrogenase is exposed to O_2 in the absence of H_2, the enzyme loses up to 90% of the activity and the loss is irreversible (Seefeldt and Arp, 1989b). In the following experiment, the *A. vinelandii* hydrogenase was exposed to O_2 alone (no H_2) util 62% of the activity was lost. The absorption spectrum again revealed increased absorption in the 530 to 300 nm region (Fig. III-2B). The difference spectrum (O_2-inactivated *minus* H_2-reduced) revealed a broad peak with a maximum near 430 nm

Figure III. 2. UV-Vis. Absorption Spectra of *A. vinelandii* Hydrogenase upon Exposure to O_2 and/or H_2. Fig. A: The H_2-activated hydrogenase (0.48 mg protein.ml^{-1}, stripped of dithionite under H_2) was scanned for absorption spectrum in an H_2-filled cuvette (——). Then the enzyme was exposed to O_2 in the H_2 (80 kPa) and O_2 (21 kPa) filled vial (8.3 ml). At 5 min, the O_2 inhibited enzyme was transferred back to the H_2-filled cuvette, and was scanned (—·—). Meanwhile, the O_2 inhibited enzyme was evacuated to remove O_2 and equilibrated with H_2 for 60 min. The enzyme was fully recovered in activity of H_2 oxidation, then the spectrum was scanned in an H_2-filled cuvette (-----). Inset shows the difference spectra of O_2 inhibited hydrogenase *minus* the H_2 activated (—·—) and the H_2 re-activated *minus* the H_2 activated hydrogenase (-----). Fig. B: The H_2-activated hydrogenase (0.28 mg protein.ml^{-1}) was scanned in the H_2 filled cuvette (——). Then, to remove the H_2, the enzyme was evacuated and equilibrated with N_2 in a 8.6 ml vial. Then 20 ml of air was injected into the vial. At 15 min after the injection of air, when 62% of the activity was lost, the spectrum was recorded in an air-filled cuvette (—·—). When 9% of activity remained (at 45 min after injection of air), the enzyme was evacuated to remove the O_2 and was incubated with H_2 for 60 min. No activity in H_2 oxidation was recovered. The absorption spectrum of this H_2 re-treated hydrogenase was recorded in the H_2-filled cuvette (-----). Inset shows the difference spectra of O_2 inactivated *minus* H_2 activated hydrogenase (—·—) and H_2 re-treated *minus* the H_2 activated hydrogenase(-----).

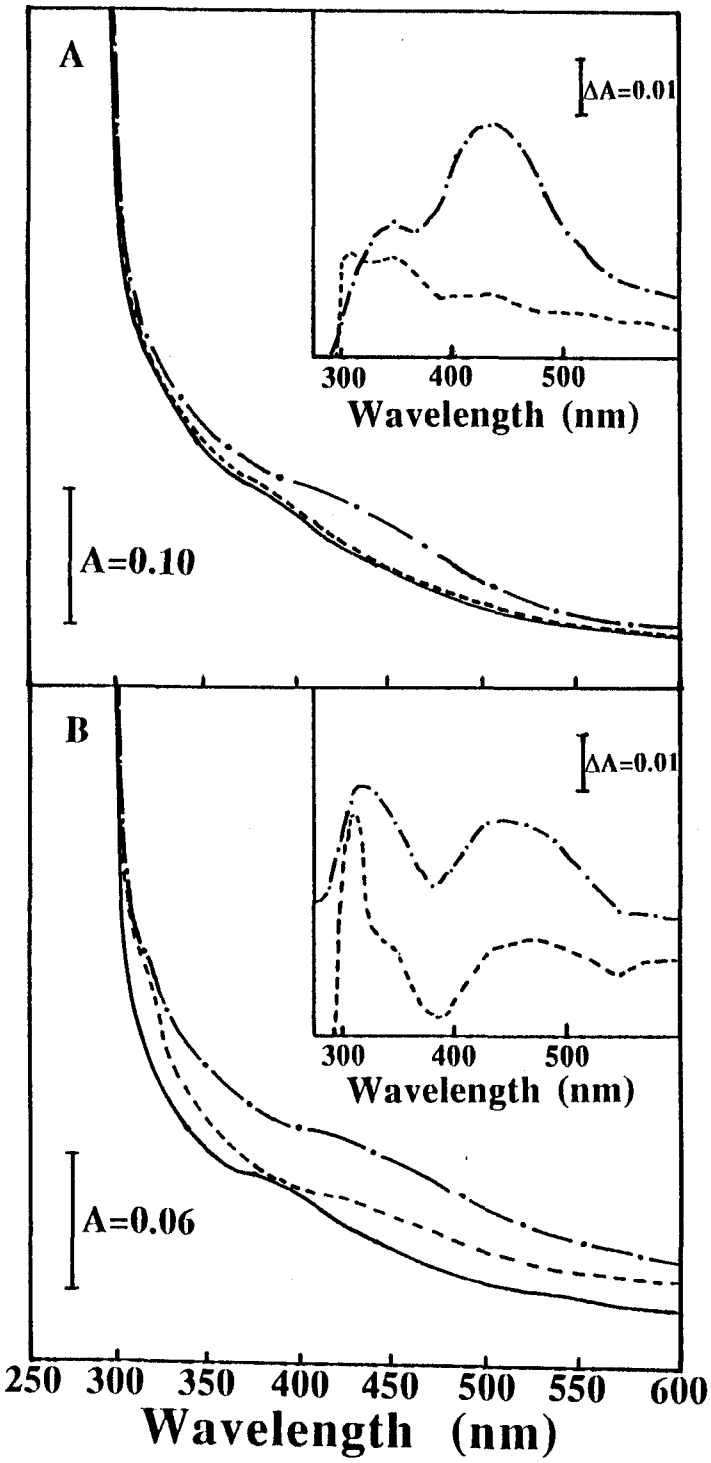
A
ΔA=0.01
300
400
500
Wavelength (nm)
A=0.10
B
ΔA=0.01
300
400
500
Wavelength (nm)
A=0.06
250 300 350 400 450 500 550 600
Wavelength (nm)

Figure III. 2.

and a narrow absorption peak at 315 nm with a shoulder at 345 nm (Fig. III-2B, inset). Removal of the O_2 and replacement with H_2 resulted in a decreasing of the broad peak at 430 nm and the 345 nm shoulder (Fig. III-2B) that was most likely due to re-reduction of [Fe-S] clusters. This is consistent with the EPR results which also indicated that the [Fe-S] clusters were still intact following irreversible loss of activity upon exposure to O_2. Upon removal of the O_2 and treatment of the enzyme with H_2, much of the g=1.94 signal returned (Jensen, et al., 1992). However, the narrow peak at 315 nm remained virtually unchanged upon exposure of the O_2-inactivated enzyme to H_2. No enzyme activity was recovered.

These results indicate that exposure of *A. vinelandii* hydrogenase to O_2 results in an oxidation of one or more of the [Fe-S] clusters. However, there are differences in the absorption spectra between the reversibly and irreversibly inactivated forms of the enzyme. Presumably, these differences reflect differences in the oxidation state of the [Fe-S] clusters or other chromophores under these different conditions.

UV-vis. Absorption Spectra of *A. vinelandii* Hydrogenase in the Presence of C_2H_2

When *A. vinelandii* hydrogenase was incubated with C_2H_2, a time-dependent change in the absorption spectrum was observed (Fig. III-3). Peaks developed simultaneously at 290 nm, with a shoulder at 235 nm, and at 492 nm. When hydrogenase was incubated under C_2H_2 plus H_2, there was no change in the spectrum above 350 nm while some small changes were apparent between 250 and 350 nm. Because hydrogenase is not inhibited by C_2H_2 when incubated in the presence of H_2, all of the change above 350 nm and most of the changes below 350 nm appear to be correlated with inhibition byC_2H_2. The time-dependency of the absorption changes were consistent with the time-dependency of C_2H_2 inhibition.

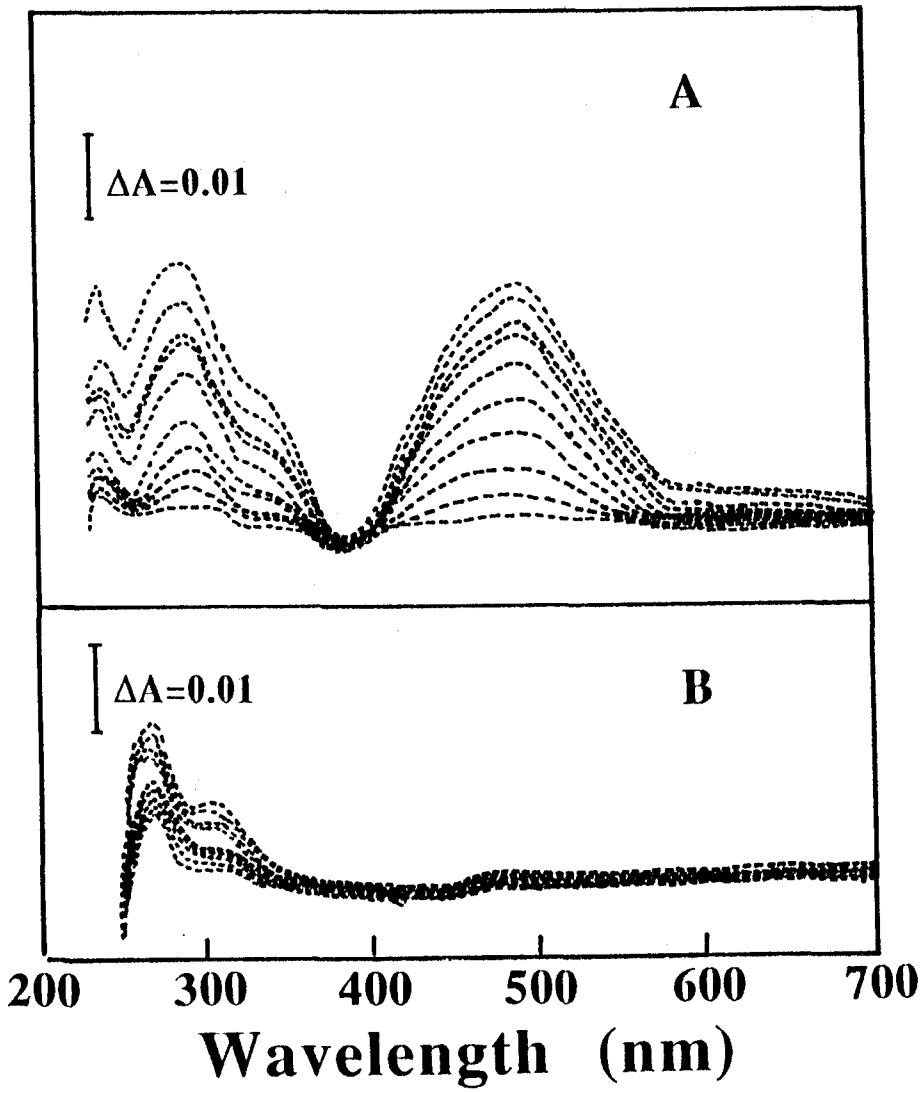

Figure III. 3. UV-vis. Absorption Spectra of C_2H_2-Treated *A. vinelandii* Hydrogenase at pH 5.0. Fig. A: The isolated hydrogenase was stripped of dithionite under N_2 at pH 5.0 (1 mM NH_4HCO_3 buffer containing 2 mg.ml^{-1} EDTA), then was scanned in the N_2-filled cuvette as background (Protein: 0.54 mg.ml^{-1}). After that, 30% of C_2H_2 was injected into the cuvette, and the difference spectra of C_2H_2-treated hydrogenase minus N_2-treated hydrogenase were recorded at 1.5, 4.5, 8.5, 15.3, 24.5, 38.5, 51.0,65.0,83.5, and 103.5 min after C_2H_2 injection Fig. B: The conditions were same as that in Fig. A, but N_2 was replaced by H_2, and the concentration of protein was 0.42 mg.ml^{-1}.

Recovery of hydrogenase activity following inhibition by C_2H_2 requires 20-50 hours, but does eventually proceed to completion. During the time course of recovery from C_2H_2 inhibition, changes in the C_2H_2-induced absorption peak at 492 nm were monitored. Because of the length of time required for recovery, it was impractical to attempt to record difference spectra. Instead, the absorption difference between 387 nm (where no absorption changes were induced by C_2H_2) and 492 nm were monitored. As the activity increased from 0 to 100 %, the Δ 492-387 decreased from its maximum to near zero (Fig. III-4). This result indicated that the absorption change associated with C_2H_2 inhibition was reversible, as was the inhibition. Furthermore, the level of activity was inversely proportional to the intensity of the absorption peak at 492 nm. The results with C_2H_2 indicate that the absorption changes induced by C_2H_2 are closely correlated with inhibition of activity by C_2H_2 and do not simply reflect a non-specific interaction of C_2H_2 with hydrogenase.

Having examined the effect of addition of O_2 on the absorption spectra of C_2H_2-treated hydrogenase, we found the absorption bands at 435 nm and 345 nm still developed upon exposure of the enzyme to O_2, and the absoptions were diminished by removal of O_2 and addition of H_2 (Fig. III-5). This result showed that the treatment with C_2H_2 of enzyme did not protect the enzyme from the oxidation of [Fe-S]centers upon incubation with O_2. In addition to this, after long-term incubation with air, the C_2H_2-pretreated hydrogenase was still able to recover activity in the presence of H_2. The effects of C_2H_2 shown here are similar to the effects of H_2. C_2H_2, like H_2, provided a protective effect on the hydrogenase from O_2-inactivation.

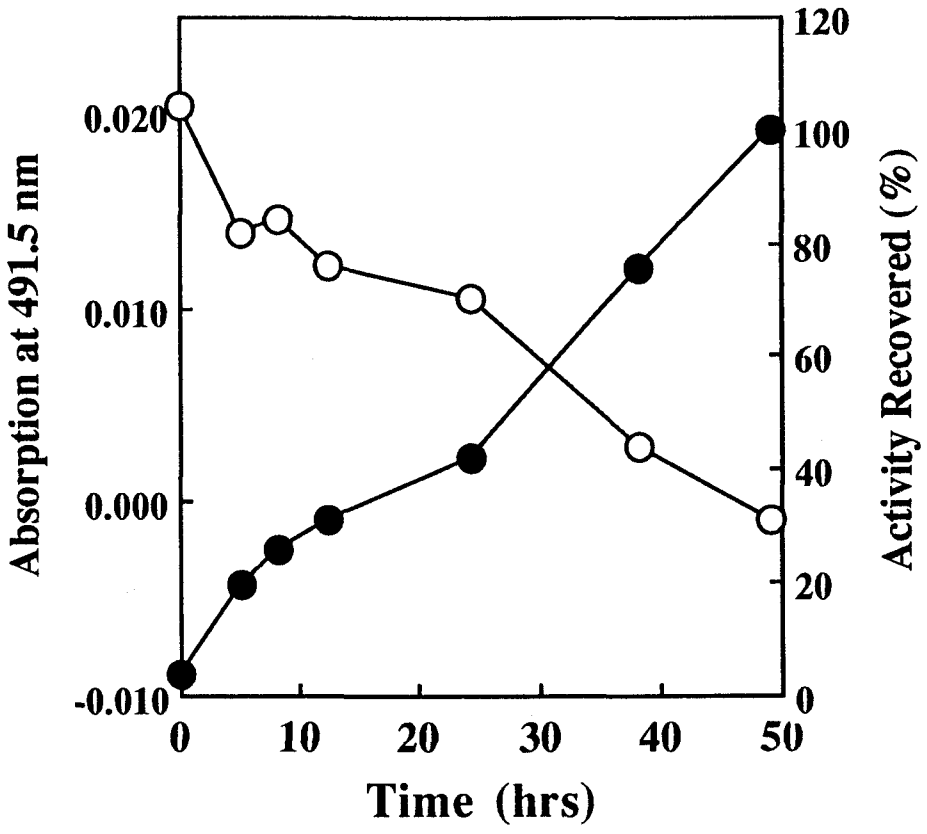

Figure III. 4. Decrease of Absorption at 491.5 nm upon Recovery of Activity of *A. vinelandii* Hydrogenase from C_2H_2 Inhibition. The C_2H_2 inhibited hydrogenase (0.37 mg. ml^{-1}, 4.5% activity remaining) was transferred to a double chamber vial that contained 101 kPa H_2. At the indicated times, the enzyme was transferred to an H_2-filled cuvette, and the difference spectra of H_2 re-activated, C_2H_2 inhibited enzyme *minus* the N_2 treated hydrogenase were recorded. At the same time, the H_2 oxidation activity was monitored by using MB reduction assay method. After scanning each sample, the enzyme was transferred back to the double chamber vial. The absorption at 491.5 nm was expressed relative to the absorption at 387 nm. The activity recovered was relative to the activity before the C_2H_2 inhibition occurring.

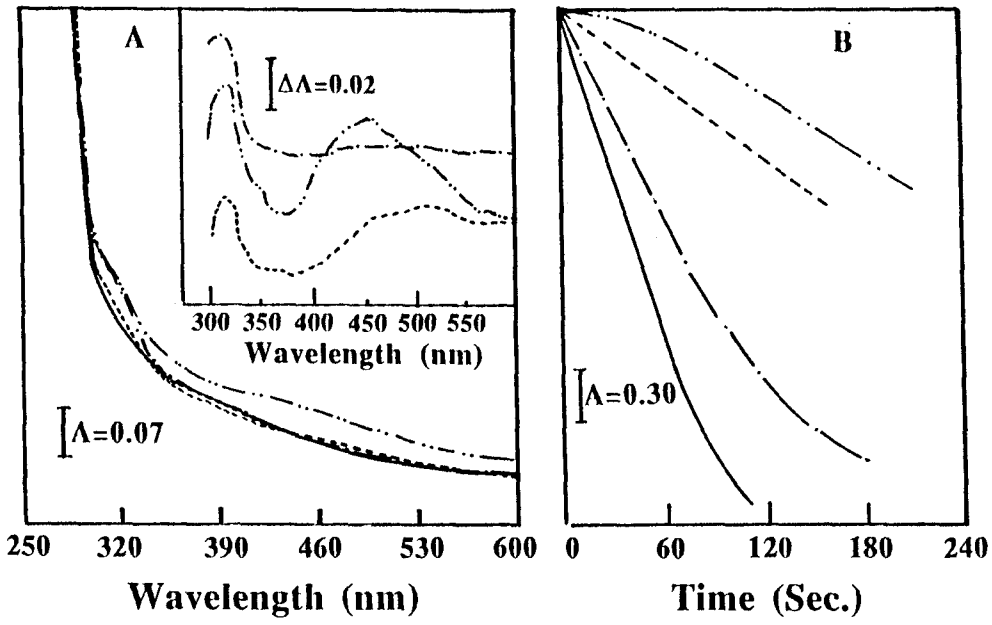

Figure III. 5. Effects of O_2 on the UV-vis. Spectra and Activities of *A. vinelandii* Hydrogenase upon Incubation with C_2H_2. The hydrogenase was stripped of dithionite under H_2 at pH 7.4 (20 mM Tris-HCl buffer containing 2 mM EDTA), and then evacuated to remove H_2 and equilibrated with N_2. The enzyme was in the active state (N_2 treated) as indicated by activity measurements (——, in Fig. B). After scanningthe spectrum in the N_2-filled cuvette (——, in Fig. A), the enzyme (0.44 mg.ml-1) was treated with C_2H_2 (100%) in the double chamber vial that contained 0.5 ml of 100 mM dithionite in the outside chamber to scavenge residual O_2. The spectrum of C_2H_2-inhibited enzyme was recorded at 15 min when about 80% of activity in the H_2 oxidation was inhibited (-----, in Fig. A & B). The C_2H_2-inhibited enzyme was further treated with O_2 in an 8.3 ml, N_2-filled vial. No inactivation was observed except for the lag in the progress curve (—··—, in Fig. B). This C_2H_2, O_2-inhibited enzyme was scanned for its absorption in a air-filled cuvette (—··—, in Fig. A). Then, the C_2H_2, O_2-inhibited enzyme was re-activated in another double chamber vial by removal of C_2H_2 and O_2 and incubation with H_2 for overnight. 62% of H_2 oxidation activity was recovered (—·—, in Fig. B). The spectrum of the H_2 re-activated enzyme was scanned in the H_2-filled cuvette (—·—, in Fig. A). The inset in Fig. A shows the difference spectra of the C_2H_2 inhibited *minus* the N_2 activated (-----), the C_2H_2 and O_2 inhibited *minus* the N_2 activated hydrogenase (—··—) and the H_2 re-activated minus the N_2 activated hydrogenase (—·—).

UV-vis. Absorption Spectra of *A. vinelandii* Hydrogenase in the Presence of CN^-

CN^- can irreversibly inactivate *A. vinelandii* hydrogenase when the enzyme is in the presence of H_2 and O_2. However, when in the active state (e.g. in the presence of H_2 alone), CN^- has no effect on hydrogenase (Seefeldt and Arp, 1989a). Consistent with these kinetic observations, there was no change in the absorption spectrum of H_2-reduced *A. vinelandii* hydrogenase when treated with cyanide (Fig. III-6B). However, upon addition of O_2 the UV-visible absorption changed (Fig. III-6B). The new spectrum was characterized by decreases in absorption in the 320 to 400 nm range and increases in the 400 to 600 nm range. Note that treatment with H_2 and O_2 in the absence of cyanide resulted in changes as well (Fig. III-2A), but the changes were not the same as those observed when cyanide was present. This point is shown more clearly in Fig. III-6A. In this case, hydrogenase was first incubated with H_2 and O_2 and the absorption spectrum was recorded. When cyanide was added to the sample, a substantial change in the spectrum was observed. The cyanide-induced spectrum was the same regardless of the order of addition of the ligands.

In neither case did replacement of the gas phase with H_2 result in a return to the "before cyanide" spectrum. Indeed, no changes in the spectrum were observed upon replacement of the gas phase with H_2. In contrast, addition of dithionite did cause some bleaching of the absorption spectrum. This result suggests that H_2 could not reduce the [Fe-S] clusters in the cyanide-inactivated hydrogenase, while dithionite could still carry out a direct reduction of the clusters. Addition of dithionite did not restore activity.

These results suggested that CN^- was interfering with the enzyme's ability to interact with H_2, but that the redox centers could still be reduced by dithionite. To provide further evidence that CN^- was interfering with the ability of hydrogenase to

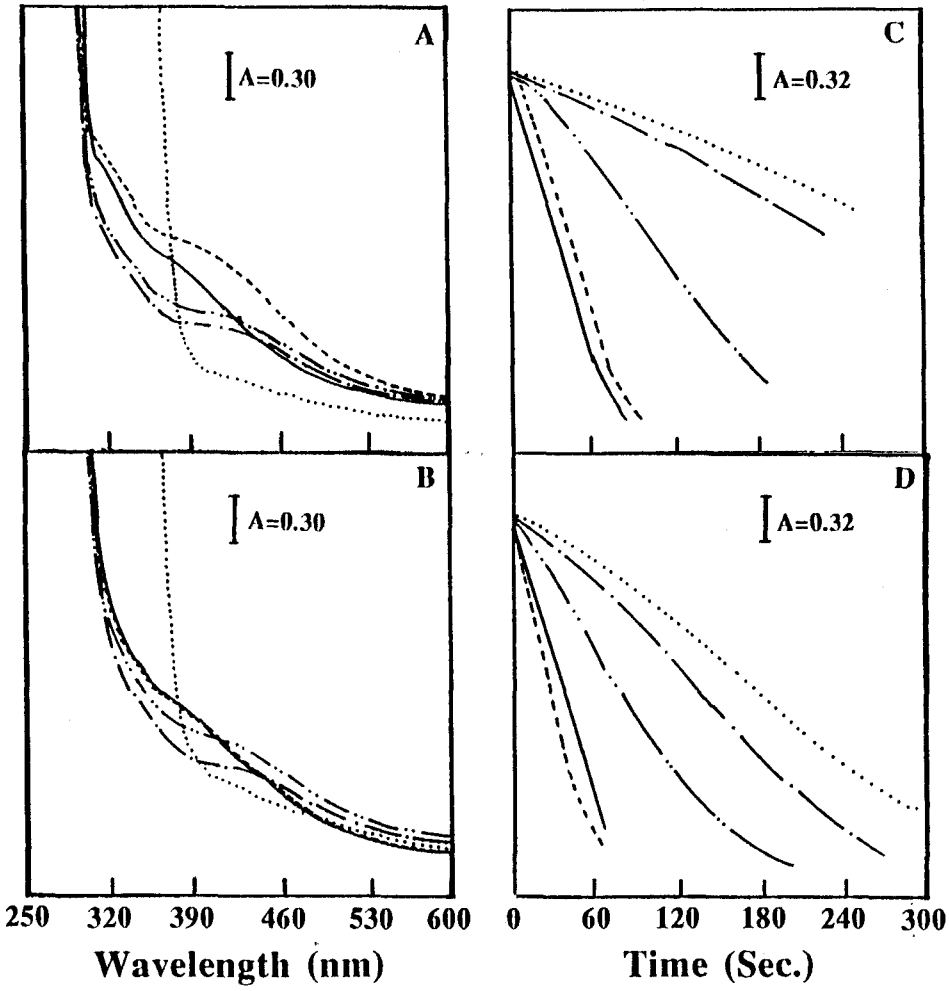

Figure III. 6. UV-vis. Spectra and H_2 Oxidation Activities of *A. vinelandii* Hydrogenase upon Treatment with CN^-. In Fig A and C, the isolated hydrogenase was stripped of dithionite under H_2 at pH 7.6 (20 mM Tris-HCl buffer) (——). Then the hydrogenase was treated with O_2 (21 kPa) for 5 min in the presence of H_2 (80 kPa) to produce the O_2-inhibited state (-----). To this O_2-inhibited hydrogenase, 10 mM anaerobic KCN was added and incubated for 30 min resulting in inactivation (—··—). Next, the CN^- inactivated hydrogenase was evacuated to remove O_2, and then incubated with H_2 for 60 min (—·—), or was incubated with 2 mM of dithionite for 30 min (·····). In Fig. B and C, the H_2 activated hydrogenase (——) was incubated with 10 mM KCN (purged with N_2) in a H_2 (101 kPa)-filled vial. After 30 min incubation, the hydrogenase remained in the active state (-----). To this CN^- containing, active hydrogenase, O_2 was added by replacement of gas phase to an O_2 (21 kPa) and H_2 (80 kPa) containing gas phase. At 30 min after introduction of O_2, the hydrogenase was inactivated (—··—). This O_2 initiated, inactive form of enzyme caused by CN^- remained inactive even after removal of O_2 by evacuation and 60 min incubation with 101 kPa H_2 (—·—), or 30 min incubation with 2 mM dithionite (·····). The hydrogenase used in these experiment contains 0.22 mg protein per ml.

interact with H_2, we determined the effect of H_2 on the isotope exchange assay catalyzed by hydrogenase. When hydrogenase (in the presence of H_2 and O_2) was treated with CN^-, the tritium exchange activity was irreversibly inactivated. The inactivation required O_2, just as the inactivation of methylene-blue linked H_2 oxidation required O_2 (Fig. III-7). Cyanide inactivation of tritium exchange followed a first order process and was dependent upon the concentration of CN^-. A bimolecular rate constant for inactivation of tritium exchange by CN^- of 12.7 $M^{-1}.min^{-1}$ was determined. This compares with the value of 23.1 $M^{-1}.min^{-1}$ determined previously for the inactivation of methylene-blue linked H_2 oxidation by cyanide (Seefeldt and Arp, 1989a).

UV-vis. Spectra of *A. vinelandii* Hydrogenase in the Presence of NO

NO, like O_2, can inhibit hydrogenase either reversibly or irreversibly. But the interaction of NO with hydrogenase was more complex than the interaction of O_2 with hydrogenase. H_2 does not prevent the irreversible inactivation by NO of the enzyme (Hyman and Arp, 1991). In the UV-vis spectra, the NO-treated hydrogenase exhibited a complicated absorption pattern (Fig. III-8). Addition of NO to the dithionite-reduced hydrogenase resulted in a broad absorption in the range of 530 to 400 nm and an increased absorption at 320 nm, in addition to the absorption of NO at 350 nm. This was very similar to that exhibited by O_2 inactivated hydrogenase. To this NO-treated hydrogenase, the addition of O_2 resulted in a dramatic decrease of absorption in the range of 340 to 530 nm and at about 320 nm (Fig. III-8A). These results indicated that the [Fe-S] clusters were destroyed by NO plus O_2. When the O_2-treated hydrogenase was exposed to NO, the solution became turbid (Fig. III-8B). After removal of O_2 and NO, a UV-vis spectrum similar to that in Fig. III-8A was observed, indicating that the [Fe-S] clusters were destroyed (Fig. III-8B).

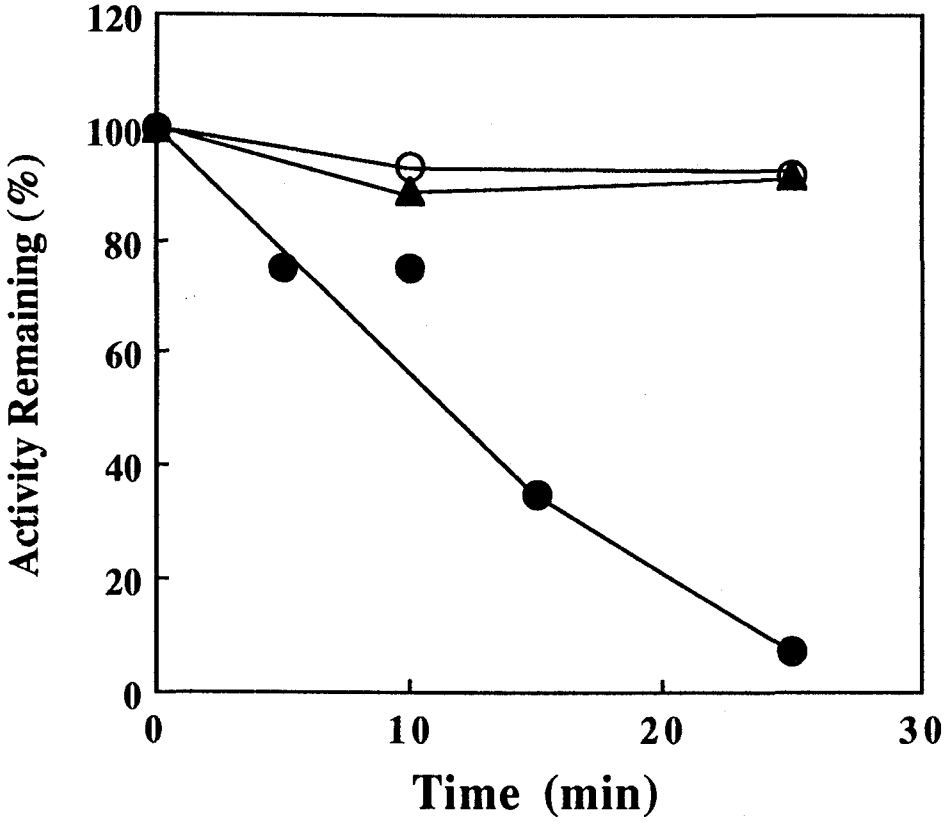

Figure III. 7. Effects of CN^- on Exchange Activity of *A. vinelandii* Hydrogenase. The purified hydrogenase (0.50 mg protein.ml^{-1}) was added to a stoppered vial containing an equal volume of 50 mM HEPES buffer (pH 7.4) and the following gas phases: (○, ●), 20 kPa O_2 and 81 kPa H_2; (▲), 101 kPa H_2. Treatments were initiated by the addition of cyanide to a final concentration of KCN of 10 mM (0.74 mM CN^-) to all vials except sample (○). At the indicated times, samples (1.49 μg protein) were removed and assayed for $^3H_2$ exchange activity (Seefeldt et al., 1986 J. Biol. Chem. 262:16816-16821). Residual activity (as a percentage of the initial activity) is plotted versus the time of incubation.

Figure III. 8. UV-Vis. Absorption Spectra of *A. vinelandii* Hydrogenase upon NO Treatments. A: The isolated, dithionite-containing hydrogenase (0.47 mg protein.ml^{-1}) (——) was incubated with NO (100%) for 5 min (-----); 6% activity remained. Then this NO inactivated hydrogenase was incubated with O_2 in a air-filled vial for 5 min and the absorption spectrum was recorded (—·—); 4% activity remained. After removal of NO and O_2 through evacuation and incubation with H_2 (101 kPa) for 30 min, no activity was detected. B: The isolated hydrogenase was stripped of dithionite under H_2. Then this H_2 treated hydrogenase (0.31 mg protein.ml^{-1}) was treated with O_2 for 7 min in the air-filled vial (——); 22% activity remained. Then this O_2 inactivated hydrogenase was transferred to a NO (100%) containing vial. After 10 min incubation, 0.04% activity remained, and the spectrum was recorded (-----). The O_2 and NO containing, inactive hydrogenase was evacuated to remove O_2 and NO and equilibrated with H_2 (101 kPa) for 30 min, then 20 kPa O_2 was added back to the enzyme. At 30 min after addition of O_2, the spectrum was again recorded.(—·—).

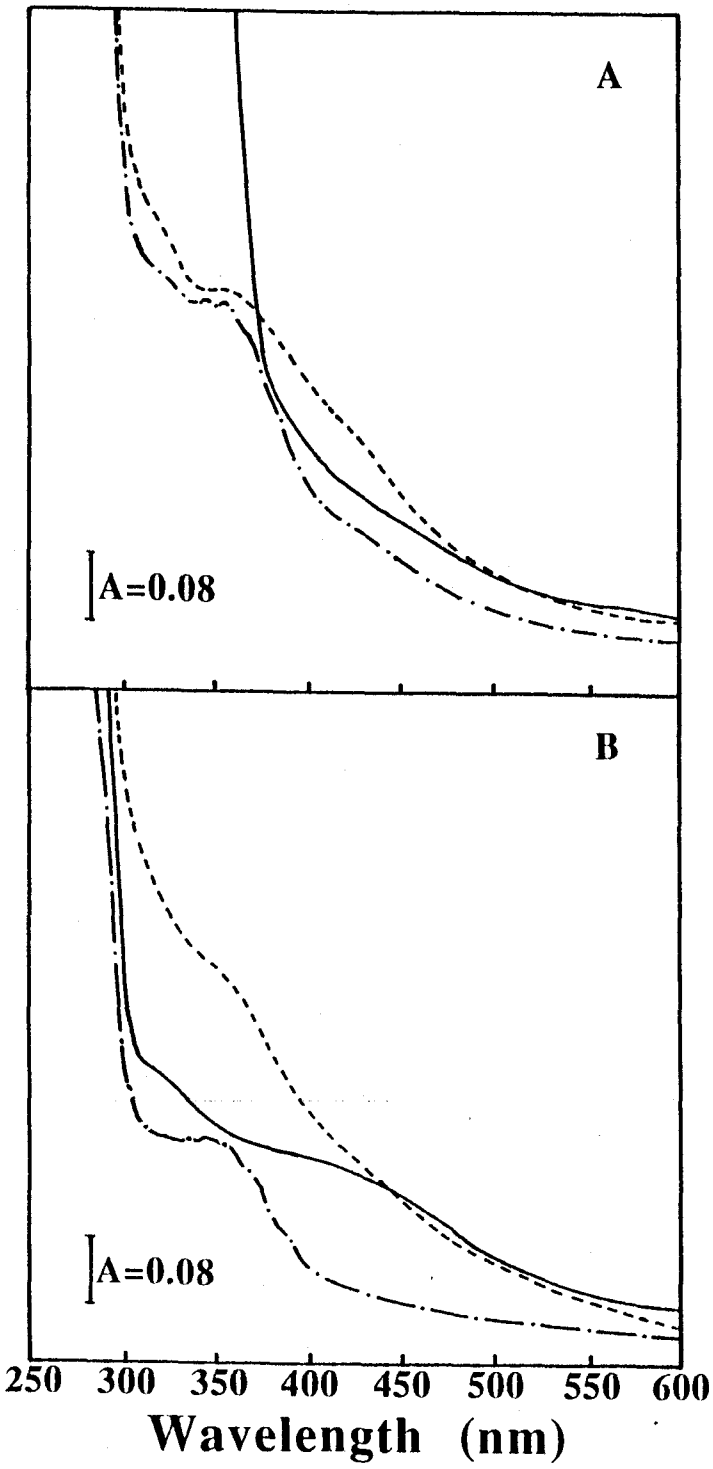

Figure III. 8.

Discussion

There remains a disagreement in the literature as to whether there is an absorption peak at 408 nm or 410 nm in the spectrum of hydrogenase. The 408 nm absorption peak was reported in the oxidized form of the hydrogenase from *Desulfovibrio vulgaris* (Haschke and Campbell, 1971, LeGall, et al., 1971, Yagi, et al., 1973); *Chromatium* (Gitlitz and Krasna, 1975); *Alcaligenes eutrophus* (Schink and Schlegel, 1979) and *Desulfovibio salexigens* (Teixeira, et al., 1986). The absorption did not decrease when the enzyme was reduced. In *Bradyrhizobium japonicum*, a 408 nm absorption peak was found in the anaerobically purified enzyme in the presence of dithionite (Arp, 1985). Thus, the 408 nm absorption peak seems likely not to arise from a prosthetic group involved in a redox reaction of hydrogenase. On the other hand, hydrogenases from other organisms, including *Clostridium pasteurianum* (Chen and Mortenson, 1974, Multani and Mortenson, 1972, Nakos and Mortenson, 1971), *Proteus mirabilis* (Schoenmaker, et al., 1979); *Desulfovibrio gigas* (Hatchikian, et al., 1978), and *Desulfovibrio desulfuricans* (Lalla-Maharajh, et al., 1983), do not exhibit the 408 nm absorption peak. In our experiments, we further purified hydrogenase by gel filtration under anaerobic conditions. The 408 nm absorption peak was not observed in the hydrogenase-containing fractions. An absorption peak at 408 nm did correspond to a single protein with MW of 27,600 as determined by SDS-PAGE. The peak at 408 nm did not decrease upon addition of H_2 or dithionite, but shifted to 415.5 nm. After exposure to O_2, the peak shifted back to 408 nm. These characteristics explain why a peak near 408 nm was observed in both the oxidized form and reduced form of some hydrogenases and not in other hydrogenases.

The further purified hydrogenase from *A. vinelandii* exhibits a continuous and increasing absorption from 600 nm to 280 nm for the reduced form, or a broad band around 435 nm with small shoulder at 325 nm for the oxidized form. Most Ni and [Fe-S] containing enzymes examined exhibit these features, including urease(Blakeley, et al.,

1983, Dixon, et al., 1975) and carbon monoxide dehydrogenase (Bonam and Ludden, 1987, Ensign, et al., 1989). In the difference spectrum of oxidized minus reduced enzyme, a large ΔA peak centered at 425-445 nm and a small ΔA peak at 325-345 nm is typical of oxidized [Fe-S] cluster in protein (Sweeney, 1980).

Effects on Spectra of Ligand Binding to Reduced *A. vinelandii* Hydrogenase

The influence of various inhibitors and the substrate, H_2, on spectral properties of *A. vinelandii* hydrogenase can be interpreted within the framework of the influence of these compounds on the catalytic properties of hydrogenase. For example, H_2, CO and C_2H_2 are competitive for binding to hydrogenase, yet their binding did not cause a marked change in the EPR signal associated with reduced hydrogenase(Seefeldt, 1989). Given that the EPR signal associated with reduced *A. vinelandii* hydrogenase can be attributed to a [4Fe-4S] center interacting with another paramagnetic species (Jensen, et al., 1992), the lack of influence on the EPR signal by these ligands indicates that they do not bind to the [4Fe-4S] cluster(s). Likewise, neither C_2H_2 nor CO had any effect on the EPR spectrum of dithionite-reduced hydrogenase isolated from *Azotobacter chroococcum* (van der Werf and Yates, 1978). The presence of H_2 also did not alter the UV-vis spectrum of *A. vinelandii* hydrogenase(Fig. III-1). Because the broad spectrum in the visible range is likely dominated by the [Fe-S] clusters, the lack of influence by H_2 supports the proposal that H_2 does not bind to these clusters. The lack of influence of H_2 on either the EPR or UV-visible spectrum of reduced hydrogenase is somewhat surprising given that H_2 can bind to and is activated by this form of the enzyme. If one of the metals is indeed involved in the binding and activation of H_2, then any changes in the metal upon binding of H_2 are not reflected in either the UV-visible or EPR spectra of *A. vinelandii* hydrogenase.

In contrast to the binding of H_2 and CO, the binding of C_2H_2 to hydrogenase did result in changes in the UV-visible spectrum of the reduced enzyme. The appearance and disappearance of the peaks correlated with the inhibition and recovery of activity (Fig. III-3). To a first approximation, the C_2H_2-inhibited *minus* reduced difference spectrum is similar to that of the O_2-oxidized *minus* reduced spectrum. However, the peaks which develop are not likely to be due to an oxidation of [Fe-S] clusters. First, the 492 nm peak was red-shifted from the peak which developed when the [Fe-S] clusters were oxidized and the UV absorption was at lower wavelengths. Furthermore, addition of O_2 to the C_2H_2-inhibited hydrogenase still resulted in the increase of absorption of [Fe-S] clusters at 435 nm. Third, the EPR spectrum of the C_2H_2-inhibited hydrogenase did not reveal an oxidation of the [Fe-S] clusters (Arp, unpublished data). One can envision two general mechanisms for the formation of the spectral changes which occur when C_2H_2 binds to hydrogenase. First, it may be that C_2H_2 binds to a component of hydrogenase to produce a new chromophore. This mechanism would require the continued binding of C_2H_2 to hydrogenase to maintain the absorption difference. Alternatively, C_2H_2 binding could induce a change in the hydrogenase (e.g. a chemical reaction or a ligand rearrangement) that produces the chromophore. This mechanism would not require the continued binding of C_2H_2 or its reaction product to hydrogenase. Clearly, the change induced by C_2H_2 would have to be reversible. With regard to the first alternative, it has been proposed that C_2H_2 binds to Ni, analogous to the proposed binding of H_2 to Ni (He, et al., 1989b, Hyman and Arp, 1987a). Certainly, there is precedent in coordination complex chemistry for formation of Ni:C_2H_2 complexes and for visible light absorption by these complexes (Nag and Chakravorty, 1980). Both π-bonded Ni:C_2H_2 complexes and Ni:acetylide complexes are possible. The latter would be analogous to the proposed formation of a Ni:hydride upon the binding of H_2 to Ni. However, C_2H_2 chemistry is remarkably diverse and other possibilities (e.g. interaction of C_2H_2 with an [Fe-S] center or other constituent of the enzyme) cannot be ruled out.

With regard to the second potential mechanism for the formation of the spectral change that occurs upon the binding of C_2H_2, Fig. III-4 shows that the loss of the absorption during recovery of activity is inversely proportional to the recovery of activity as a function of time. We have recently demonstrated that C_2H_2 release from inhibited hydrogenase is not proportional to recovery of activity, rather C_2H_2 release precedes recovery of activity. This observation, coupled with the results of Fig. III-4, suggest that the absorption change does not require that C_2H_2 be bound to the protein, but rather that the change is a result of the binding of C_2H_2. It should also be noted that upon activation of aerobically purified *A. vinelandii* hydrogenase, the first change which occurs in the UV-vis spectrum is the loss of absorption at about 490 nm which is subsequently followed by a loss of absorption centered at 430 nm (Sun and Arp, 1991).

Acetylene interacts with a number of metalloenzymes in addition to hydrogenase (Hyman and Arp, 1988). The modes of interaction include acting as an inhibitor (e.g. N_2O reductase), mechanism-based inactivator (e.g. ammonia monooxygenase, methane monooxygenase) and substrate (e.g. nitrogenase). However, we are not aware of any other cases where binding of C_2H_2 to the enzyme results in the occurrence of a spectral change comparable to that observed when C_2H_2 binds to hydrogenase.

Effects on Spectra of Ligand Binding to Oxidized Hydrogenase

When hydrogenase was incubated with O_2, either in the presence or absence of H_2, the iron sulfur centers were oxidized as evidenced by changes in the EPR signals (Jensen, et al., 1992) and changes in the UV-vis spectra (Fig. III-2). Both the EPR and UV-visible spectra of hydrogenase in the O_2-oxidized state were influenced by the presence of H_2 which is consistent with the influence of H_2 on the stability of O_2-oxidized hydrogenase. While it would appear from both the EPR and the UV-visible spectra that the [Fe-S] clusters are influenced by the presence of H_2, a detailed

description of the nature of the interaction is not yet possible. As expected given the reversibility of the inhibition by O_2 in the presence of H_2, the oxidized absorption spectrum (Fig. III-2A) and EPR spectrum (Jensen, et al., 1992) were completely reversed to the corresponding reduced spectra upon removal of O_2. In the presence of O_2 alone, the loss of activity is irreversible. Nonetheless, the [Fe-S] clusters can be re-reduced by H_2 (the hydrogenase retains ca. 10% activity following even prolonged exposure to O_2) (Fig. III-2B). This result confirms a conclusion based originally on of EPR data, namely, that the loss of activity caused by exposure to O_2 does not result in a loss of the [Fe-S] clusters.

In the absence of H_2, the O_2 oxidized hydrogenase showed a ΔA peak at 315 nm that did not decrease upon incubation with H_2. This absorption seemed to correspond to the O_2 inactivation, but a detailed description of the moleculor assignment of this peak was not available. Two kinds of Ni-compounds show absorption peaks near 315 nm (See (Blakeley, et al., 1983)). The first one is Ni(II)-thiolate complexes which have an intense RS-Ni(II) charge transfer absorption peak at about 300 nm and a broad absorption peaks at wavelengths greater than 450 nm which is grossly consistent with d-d ligand field transitions. The second one is complexes of Ni(III) with tripeptides, which have an intense charge-transfer absorption peak near 330 nm. Ni(II) and Ni(III) have been reported to exist in different active state of hydrogenase from *D. gigas* during the activation process (Teixeira, et al., 1989). Thus in the hydrogenase, Ni(II) could be oxidized to Ni(III) upon the irreversible O_2 inactivation, resulting in an absorption peak at 315 nm. An alternative possibility is that the ΔA peak at 315 nm is due to the RS-Ni(II) charge transfer absorption. The RS-Ni(II) charge transfer could be induced by either oxidation of Ni(I) or oxidation of RSH group.

Cyanide inactivates only oxidized hydrogenase(Seefeldt and Arp, 1989a); spectral changes associated with the binding of cyanide to hydrogenase were consistent with this

observation. No changes in the UV-visible spectrum were observed when reduced hydrogenase was incubated with cyanide while incubation of the H_2- and O_2-treated hydrogenase with cyanide resulted in marked absorption changes (Fig. III-6). When treated with dithionite, some bleaching of the UV-vis spectrum was observed, consistent with re-reduction of the [Fe-S] clusters. H_2 was not able to bleach the spectrum, indicating that CN- blocked the H_2-activating site. Cyanide is a common inhibitor of metalloenzymes, but there are relatively few cases where cyanide has been shown to inhibit hydrogenases. Most trials, however, have not considered the redox state of the hydrogenase. In the case of *A. vinelandii*, the spectral studies do not reveal which redox center in the oxidized hydrogenase binds cyanide. One potential mechanism for the interaction of cyanide with hydrogenase is that cyanide binds directly to Ni resulting in the formation of Ni:cyano complex. The requirement that hydrogenase be oxidized before cyanide can bind could indicate that cyanide binds only to a more oxidized state of the Ni. However, an oxidation of Ni was not apparent as a redox-dependent change in a Ni signal in the EPR spectrum. Furthermore, a change in oxidation state for Ni is not necessary to accommodate the binding of cyanide as this ligand can bind to Ni in several oxidation states, including I, II, and III (Coyle and Stiefel, 1988). Regardless of the oxidation state of the Ni, cyanide binding to Ni might well be accompanied with a UV-visible absorption change. However, any such change will be difficult to separate from the [Fe-S] cluster absorption. It might also be that cyanide binds to an [Fe-S] cluster. However, there is only one report of the binding of cyanide to an iron sulfur center (Conover, et al., 1991) and in that case one of the four cysteines that usually binds [Fe-S] clusters to proteins was not present, thereby providing a binding position for cyanide. Perhaps oxidation of hydrogenase also results in the formation of an Fe on an [Fe-S] cluster that is not fully coordinated, thereby creating a binding site for cyanide.

The decrease in absorption in the region of 460-290 nm which resulted from CN^- inactivation was exhibited as a trough in the difference absorption spectrum of CN^-

treated enzyme versus active enzyme. This trough is located at 336 nm prior to the initiation of CN^- inactivation but shifts to 380 nm upon the addition of CN^-. Similar phenomenon has been reported in xanthine dehydrogenase, a molybdenum-enzyme. Inactivation by CN^- of the xanthine dehydrogenase enzyme resulted in a major trough at 320 nm in the difference spectrum between CN^- treated and native enzyme. That trough was explained as result from binding of Mo center with CN^- (Coughlan, et al., 1969). Cyanide serves as a strong ligand to most transition metals such as iron, nickel, copper, zinc, selenium, and molybdenum (Chadwick and Sharpe, 1986). Therefore, the trough at 380 nm could result from the binding of CN^- to the Ni center or [Fe-S] cluster(s). Cyanide can also bind to sulfur ligands and release the sulfur as thiocyanate (Solomonson, 1981). However, Seefeldt and Arp (1989) have found that [^{14}C] cyanide remained associated with the inactive enzyme during gel filtration. Consistent with this, after gel filtration the trough in the difference spectrum relative to CN^- inactivation still existed (date not shown). This implied that no SCN- was released. Thus CN did not bind to a sulfur ligand. From the absorption spectrum or difference spectrum, it seems possible that cyanide was able to slowly bleach the iron-sulfur center just like what happened in clostridial ferredoxin (Wallace and Rabirowitz, 1971). In the absorption spectrum, the absorption intensity of the oxidized [Fe-S] centers decreased with time, although the absorption shape of oxidized [Fe-S] clusters (the broad absorption band centered at 425-435 nm) was still able to be seen (Fig. III-6). It is worth noting that the bleaching of [Fe-S] clusters took place after CN^- inhibition. This implied that cyanide inactivation was not due to destruction of [Fe-S] centers.

Summary

The results presented in this manuscript support the following conclusions. 1) The influence of inhibitors and the substrate, H_2, on UV-visible spectra are interpretable

within the framework of the effects of these compounds on catalysis by hydrogenase. 2) The binding of C_2H_2 to hydrogenase results in a new UV-vis absorption feature. A similar feature has not been observed upon the binding of C_2H_2 to other metalloenzymes inhibited by C_2H_2. 3) Exposure of hydrogenase to O_2 results in an oxidation of, but not destruction of, the [Fe-S] clusters. 4) Simultaneous binding of H_2 and O_2 also results in an oxidation of the [Fe-S] clusters, but the presence of H_2 influences UV-visible spectra. 5) Cyanide-induced changes in UV-visible spectra are consistent with the catalytic effects of cyanide. Part of the resultant UV-visible spectrum may be a Ni:cyano complex.

References

Adams, M. W. W. (1990) *Biochim. Biophys. Acta 1020*, 115-145.

Arp, D. J. (1985) *Arch. Biochem. Biophys. 237*, 504-512.

Blakeley, R. L., Dixon, N. E. & Zerner, B. (1983) *Biochim. Biophys. Acta. 744*, 219-229.

Bonam, D. & Ludden, P. W. (1987) *J. Biol. Chem. 262*, 2980-2987.

Cammack, R., Fernandez, V. M. & Schneider, K. (1988) in *Bioinorganic Chemistry of Nickel* (J. R. Lancaster, Ed.) pp 167-190, VCH Publishers, New York.

Chadwick, B. M. & Sharpe, A. G. (1986) in *Advances in Inorganic Chemistry and Radiochemistry* (H. J. Emeleus and A. G. Sharpe, Ed.) pp 83-176, Academic Press Inc., New York.

Chen, J. S. & Mortenson, L. E. (1974) *Biochim. Biophys. Acta. 371*, 283-298.

Conover, R. C., Park, J.-B., Adams, M. W. W. & Johnson, M. K. (1991) *J. Am. Chem. Soc. 113*, 2799-2800.

Coughlan, M. P., Rajagopalan, K. V. & Handler, P. (1969) *J. Biol. Chem. 244*, 2658-2663.

Coyle, C. L. & Stiefel, E. I. (1988) in *The bioinorganic chemistry of nickel* (J. Jack R. Lancaster, Ed.) pp 1-28, VCH, Weinheim.

Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B. (1975) *J. Am. Chem. Soc. 97*, 4131-4133.

Eidsness, M. K., Scott, R. A., Prickril, B., DerVartanian, D. V., LeGall, J., Moura, I., Moura, J. J. G. & Perk, H. D. J. (1989) *Proc. Natl. Acad. Sci. USA 86*, 147-151.

Eisbrenner, G. & Evans, H. J. (1983) *Annu. Rev. Plant Physiol. 34*, 105-136.

Ensign, S. A., Bonam, D. & PW., L. (1989) *Biochemistry 28*, 4968-4973.

Gitlitz, P. H. & Krasna, A. I. (1975) *Biochemistry 14*, 2561-2568.

Haschke, R. H. & Campbell, L. L. (1971) *J. Bacteriol. 105*, 249-258.

Hatchikian, E. C., Bruschi, M. & J., L. (1978) *Biochem. Biophys. Res. Commun. 82*, 451-461.

He, S. H., Teixeira, M., LeGall, J., Patil, D. S., Der Vartanian, D. V., Huynh, B. H. & Perk, H. D. J. (1989a) *J. Biol. Chem. 264*, 2678-2682.

He, S. H., Woo, S. B., Der Vartanian, D. W., LeGall, J. & Peck, H. D., Jr. (1989b) *Biochem. Biophys. Res. Commun. 161*, 127-133.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1988) *Anal. Biochem. 173*, 207-220.

Hyman, M. R. & Arp, D. J. (1987a) *Biochemistry 26*, 6447-6454.

Hyman, M. R. & Arp, D. J. (1987b) *Appl. Environ. Microbiol. 53*, 298-303.

Jensen, G. M., Seefeldt, L. C., Arp, D. J. & Stephens, P. J. (1992) *Biochemistry Submitted*,

Lalla-Maharajh, W. V., Hall, D. O., Cammack, R., Rao, K. K. & LeGall, J. (1983) *Biochem. J. 209*, 445-454.

LeGall, J., Dervartanian, D. V., Spilker, E., Lee, J. P. & Perk, H. D., Jr. (1971) *Biochim. Biophys. Acta. 234*, 525-530.

Menon, A. L., Stults, L. W., Robson, R. L. & Mortenson, L. E. (1990) *Gene 96*, 67-74.

Multani, J. S. & Mortenson, L. E. (1972) *Biochim. Biophys. Acta. 256*, 66-70.

Nag, K. & Chakravorty, A. (1980) *Coordination Chemistry Reviews 33*, 87-147.

Nakos, G. & Mortenson, L. E. (1971) *Biochemistry 10*, 2442-2449.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1992) *FEMS Microbiology Reviews 88*, 109-135.

Schink, B. & Schlegel, H. G. (1979) *Biochim. Biophys. Acta. 567*, 315-324.

Schoenmaker, G. S., Oltmann, L. F. & Stouthamer, A. H. (1979) *Biochim. Biophys. Acta. 567*, 511-521.

Seefeldt, L. C. (1989) Ph. D thesis, University of California, Riverside.

Seefeldt, L. C. & Arp, D. J. (1989a) *J. Bacteriol. 171*, 3298-3303.

Seefeldt, L. C. & Arp, D. J. (1989b) *Biochemistry 28*, 1588-1596.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Seefeldt, L. C., Fox, C. A. & Arp, D. J. (1986) *J. Biol. Chem. 261*, 10688-10694.

Solomonson, L. P. (1981) in *Cyanide in Biology* (B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing, Ed.) pp 11-28, Academic Press, Inc., New York.

Sun, J.-H. & Arp, D. J. (1991) *Arch. Biochem. Biophys. 287*, 225-233.

Sun, J. H., Hyman, M. R. & Arp, D. J. (1992) *Biochemistry 31*, 3158-3165.

Sweeney, W. V. (1980) *Ann. Rev. Biochem. 49*, 139-161.

Teixeira, M., Moura, I., Fauque, G., Czechowski, M., Berlier, Y., Lespinat, P. A., LeGall, J., Xavier, A. V. & Moura, J. J. G. (1986) *Biochimie. 68*, 75-84.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., LeGall, J., Der Vartanian, D. V., Peck, H. D., Jr. & Huynh, B. H. (1989) *J. Biol. Chem. 264*, 16435-16450.

van der Werf, A. N. & Yates, M. G. (1978) in *Hydrogenases: Their catalytic activity, structure and function* (H. G. Schlegel and K. Schneider, Ed.) pp 307-326, Erich Goltze KG.

Wallace, E. F. & Rabirowitz, J. C. (1971) *Arch. Biochem. Biophys. 146*, 400-409.
Yagi, T., Kimura, K., Daidoji, H., Sakai, F., Tamura, S. & Inokuchi, H. (1973) *J. Biochem. 79*, 661-677.

Zirngibl, C., Van Dongen, W., Schwoerer, B., Von Buenau, R., Richter, M., Klein, A. & Thauer, R. K. (1992) *Eur. J. Biochem. 208*, 511-520.

CHAPTER IV

INACTIVATION OF HYDROGENASE FROM *AZOTOBACTER VINELANDII* BY COPPER

by

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Running Title: Cu(II) Inactivation Of *Azotobacter vinelandii* Hydrogenase

Contributions

Jin-hua Sun participated in developing the experimental design and the techniques utilized to perform this study, performing the experiments, analyzing the data and calculating the results. Dr. Daniel J. Arp participated in the experimental design, supervising progress of study and editing the manuscript.

Abbreviations

BSA; bovine serum albumin

DTT; dithiothreitol

EDTA; ethylenediaminetetraacetic acid

EPR; electron paramagnetic resonance

EXAFS; extended X-ray absorption fine structure

LMCT; Ligand to metal charge-transfer

MES; 2-(N-morpholino)ethanesulfonic acid

Tris; tris(hydroxymethyl)aminomethane

UV-vis.; ultraviolet-visible

Abstract

The effect of Cu(II) on the activity of *Azotobacter vinelandii* hydrogenase has been studied. Cu(II) (1 to 100 μM) irreversibly inactivated hydrogenase either under catalytic turnover condition or when incubated in the absence of a substrate. Among H_2 oxidation, H_2 production and D_2/H^+ isotope exchange reactions, no major difference was observed in terms of sensitivity to Cu(II) inactivation. The Cu(II) inactivation was dependent upon the activity state of the hydrogenase. Under turnover conditions, the inactivation constant was 10 times higher than that under nonturnover condition. Cu(II) inactivated anaerobically purified hydrogenase when in the presence of H_2 or $Na_2S_2O_4$, but not in the presence of N_2 only unless H_2 was added. The Cu(II) had little effect on the non-activated, aerobically purified hydrogenase, in which the catalytic site was not fully ready for catalysis. Cu(II) did not inactivate the C_2H_2-inhibited hydrogenase, where the H_2 activation site was presumably bound with C_2H_2. In the presence of 101 kPa CO, an active-site directed, fast binding inhibitor of hydrogenase, the Cu(II) was unable to fully inactivate the enzyme. The effects of Ni(II), Fe (II), Fe(III), Co(II), or Zn(II) on the Cu(II) inactivation have been studied. The hydrogenase could be inhibited by the Ni(II), Fe(III), or Zn(II) under turnover condition. However, only Ni(II) prevented the Cu(II) inactivation. These results demonstrate that the active state of H_2 activation site of hydrogenase is required for the Cu(II) inactivation. During the Cu(II) inactivation, the absorption of light by the [Fe-S] clusters was bleached while the absorption at 300 nm and 320 nm increased. The kinetic study indicated that the Cu(II) inactivation was a saturable process with a slow binding mechanism.

Introduction

Hydrogenases are metalloenzymes that catalyze the activation of H_2. This activation can lead to oxidation or evolution of H_2. It has been proposed that the H_2 activation must include 5 steps (Adams, 1990). Step 1 involves the diffusion of H_2 into the hydrogenase and binding to the H_2 activation site. In step 2, a polarization occurs between two H atoms, and the H-H bond breaks heterolytically. Consequently, one H atom obtains two electrons, and another H atom becomes a proton that is exchangeable with protons in the solvent. In step 3, electrons from H atom are transferred to the H_2 activation site. In return, the activation site is reduced. In step 4, some intra-molecular electron carriers (possibly [4Fe-4S] clusters) accept the electrons from the activation site and transfer the electrons to an external electron acceptor. The activation site is returned to the oxidized state. In step 5, the second proton dissociates from the activation site and the enzyme is returned back to its original state and enters another catalytic cycle. The reverse of step 5 and 4 will lead to H_2 production.

The hydrogenases isolated from several microorganisms are composed of two subunits (ca. 30 and 60 kDa) and contain Ni and two or more iron-sulfur clusters as prosthetic groups (see Chapter I). The Ni is located on the large subunit of the enzyme (Przybyla, et al., 1992, Sun, et al., 1992). Several experimental results suggested that it serves as the site of hydrogen binding and oxidation (Przybyla, et al. 1992; Saint-Martin, Lespinat et al. 1988) but conclusive evidence is still absent. The environment of the Ni has not been elucidated as yet. The EXAFS indicated that S, and N(O) atoms ligated to the Ni (Maroney, et al., 1990, Scott, et al., 1984). The [4Fe-4S] clusters were believed to react as the electron transfer carriers (Przybyla, et al., 1992). They could be located on the small subunit of the enzyme. However, no evidence can be found in the literature. Therefore, the location and function of these prosthetic groups in the H_2 activation reaction are still unknown.

Inhibitors provide a means of investigating the mechanism of the H_2 oxidation by hydrogenase and of probing the roles of the metal centers in catalysis. A number of inhibitors of *A.zotobacter vinelandii* hydrogenase have now been characterized, including O_2 (Seefeldt and Arp, 1989), CN^- (Seefeldt and Arp, 1989), C_2H_2 (Hyman and Arp, 1987a, Sun, et al., 1992) and NO (Hyman and Arp, 1991). This paper deals with the inhibitor Cu(II).

In 1954, Schlegel demonstrated a direct inhibition by Cu(II) of the hydrogen metabolism of the Knallgas bacteria *Hydrogenomonas* (Schlegel, 1954). Since then, several publications described the Cu(II) inhibition of hydrogenase. In summary: (1) Not all hydrogenase are sensitive to Cu(II) inhibition. The hydrogenase from the green sulfur bacterium *Chlorobium limicola* was only slightly inhibited by 0.5 mM Cu(II) in the H_2 uptake assay (Serebryakova, et al., 1987). (2) In some cases, the inhibitory effects were only observed in purified hydrogenase. The hydrogenase of extracts of *Desulfotomaculum orientis* was inhibited by incubation with Cu(II), but inhibition did not occur with whole cells (Cypionka and Dilling, 1986). (3) Detailed studies have been performed in purified hydrogenase from *Desulfovibrio. gigas* (Fernandez, et al., 1989). Interestingly, the Cu(II) more effectively inhibited H_2 oxidation than isotope exchange and the [4Fe-4S] clusters were destroyed.

In this paper, we report that the *A. vinelandii* hydrogenase is extremely sensitive to Cu(II) inactivation. This hydrogenase could be inactivated by μM levels of Cu(II). The inactivation caused by Cu(II) has a similar effects on the H_2 oxidation, H_2 production and isotope exchange reactions. During the Cu(II) inactivation, in addition to the bleaching of absorption of [Fe-S] clusters, absorption bands that were designated to the imidazole-Cu(II) charge transfer were observed in the UV-vis region.

Materials and Methods

Materials

H_2 and N_2 (>99.99% purity, from Liquid Carbonic Corp., Chicago, IL) were stripped of residual O_2 by passage over a heated, copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). Ultrapure $CuCl_2$ (99.999%) was obtained from Aldrich. Acetylene was further purified cryogenically as described (Hyman and Arp, 1987b). All other chemicals were of reagent grade.

Purification of *A. vinelandii* Hydrogenase

All experiments were carried out with highly purified hydrogenase. Cells of *A. vinelandii* (strain OP) were cultured and membranes were prepared as described (Seefeldt and Arp, 1989). The hydrogenase was purified from membranes as previously described (Sun and Arp, 1991). All steps were performed under anaerobic conditions and in the presence of 2 mM $Na_2S_2O_4$. For aerobic purification of *A. vinelandii* hydrogenase, the protocol was identical to that used in the anaerobic purification, except that 2 mM $Na_2S_2O_4$ was not included in the buffer and the buffer were not purged to remove O_2. Protein concentrations were determined by the Biuret method and the modified Lowery method (Peterson, 1977); BSA was used as a protein standard.

Hydrogenase Activity Assays

Reduction of methylene blue coupled to H_2 oxidation was used as the normal determination of hydrogenase activity (Sun and Arp, 1991). Progress of reactions was monitored at 690 nm with a Beckman DU-70 spectrophotometer. In addition, as required

in experiments, the H_2 evolution and D_2/H_2O exchange catalyzed by hydrogenase were also used. The methyl viologen supported H_2 evolution was determined with a Clark style electrode in 0.1M succinate buffer (pH5.0) as described (Seefeldt and Arp, 1986). The exchange reaction mixture with D_2 was assayed in a quadrupole gas analyzer (Dycor; Ametek Thermox Instruments Division, Philadelphia, Pa). The exchange reaction was carried out at room temperature in a 5-ml sealed vial under anaerobic conditions in a 1 ml of solution containing 0.05 M MES buffer (pH 6.0), 2 mM $Na_2S_2O_4$ with vigorous stirring. 1 ml of D_2 was injected into gas phase, then followed by injection of 50 μl of hydrogenase (0.054mg/ml) to initiate the exchange reaction. The rates of change in quantity for the masses of H_2, HD, and D_2 (2, 3, and 4, respectively) were monitored for at least 45 min. To normalize the quantities and ensure the absence of O_2 in the assay vial, the masses of N_2 and O_2 (28 and 32, respectively) were also monitored.

Incubation Procedure for Cu (II) Inactivation under Non-Turnover Condition

One of two procedures was used in the experiment: (1) For time-dependent, kinetic analysis of inactivation, the activity assay cuvette containing 0.8 ml of MES buffer (pH 6.0) and $CuCl_2$ was purged with H_2 for 10 min. Then hydrogenase (nM level of final concentration) was injected into the cuvette, and incubated at room temperature. At designated times, 0.2 ml of anaerobic methylene blue solution (1mM, containing 2 mM EDTA) was added. Immediately, the cuvette was inserted in a Beckman DU-70 spectrophotometer for monitoring the activity of hydrogenase. To observe the Cu (II) inactivation under nonturnover condition without presence of H_2, the cuvette was purged with N_2 for 10 min. Residual activity of enzyme was determined by addition of 2 ml of H_2 into gas phase of the cuvette. The activity from the cuvette without Cu(II) was chosen as 100%. (2) For normal analysis of inactivation, incubations were carried out in shortened test-tubes (0.5 ml volume) placed in serum vials (10 ml) sealed with butyl

rubber caps and crimped aluminum seals (Wheaton Scientific, Millville, NJ). The vials were evacuated and filled with H_2 or N_2, then anaerobic MES buffer (0.05 M, pH 6.0) or Tris buffer (0.02 M, pH 7.4), and $CuCl_2$ were injected into the shortened test-tubes. Each vial also contained an O_2 scavenger (0.5 ml of 0.1 M $Na_2S_2O_4$ in 0.1 M Tris-HCl, pH 7.5) outside the test-tube. Incubations were initiated by addition of hydrogenase to the test-tube. After 5 min, the sample was transferred to an activity assay cuvette that contained 1ml of 0.05 M MES buffer (pH 6.0), 0.2 mM methylene blue, and 2 mM EDTA.

C_2H_2 Protective Study

The C_2H_2-inhibited hydrogenase was obtained by incubations of hydrogenase with C_2H_2 as indicated (Sun et al., 1992). Then the enzyme was repeatedly evacuated to remove the free C_2H_2 and incubated with 0.5 mM $CuCl_2$ for 40 min. The incubation of non-C_2H_2 treated enzyme with $CuCl_2$ indicated that 58% of the activity was inactivated. After this, the C_2H_2-inhibited enzyme, the C_2H_2-inhibited and Cu(II)-treated enzyme, and only Cu(II) treated enzyme were desalted by Sephadex G-25 (equilibrated with 0.2 M Tris-HCl (pH 7.6) containing 2 mM EDTA and 2 mM $Na_2S_2O_4$) to remove the free Cu(II). Then all these enzyme samples were brought to activity recovery system to monitor the protective effect of C_2H_2 from the Cu(II) inactivation.

The recovery system consisted of a double-chambered vial which contained 101 kPa H2. The outer section of the vial contained an O_2 scavenger (see above). The inhibited hydrogenase was transferred to the inner chamber. The $Na_2S_2O_4$ concentration in the enzyme sample was raised to 4 mM by addition of $Na_2S_2O_4$ from a stock solution (0.1 M). At the indicated times, a sample of the enzyme was removed from the incubation vial and either assayed for hydrogenase activity.

UV-vis. Difference Absorption Spectra

Spectra were recorded with a Beckman DU-70 spectrophotometer. The protein samples were stripped of the $Na_2S_2O_4$ by gel filtration chromatography under H_2, and then transferred via gas-tight syringe to a stoppered, anaerobic, quartz cuvette containing 101 kPa H_2 or N_2. This sample was used as background. The addition of $CuCl_2$ was followed by scanning absorption from 200 to 700 nm at designated times.

Results

Inactivation of *A. vinelandii* Hydrogenase by Copper

With D. *gigas* hydrogenase Cu(II) inactivated H_2 oxidation more rapidly than isotope exchange which indicated that electron transfer from the H_2 activation site to the electron acceptor was disrupted prior to an effect on the H_2 activation site (Fernandez, et al., 1989). We tested the effects of Cu(II) on H_2 production and H_2 oxidation reactions catalyzed by *A. vinelandii* hydrogenase. When the anaerobically purified hydrogenase was incubated with 1 to 10 μM $CuCl_2$ for 30 min, its ability to either oxidize or produce H_2 decreased to a similar extent (Table IV-1). Furthermore, when we introduced μM $CuCl_2$ to the H_2 oxidation and D_2/H^+ exchange reaction vials the activities of both reactions were inhibited under turnover condition (Fig. IV-1). These results indicated that the catalytic step affected by the treatment with Cu(II) was the H_2 activation.

Table IV. 1. Effect of Cu(II) on the H_2 Oxidation and H_2 Production Catalyzed by *Azotobacter vinelandii* Hydrogenase.

$CuCl_2$ (μM)	Remaining Activity (%)	
	H_2 oxidation	H_2 production
0	100	100
1	97	74
2	62	79
5	26	38
10	27	35

Hydrogenase was incubated with $CuCl_2$ in the double chamber vials as described in the Material and Methods. At 60 min after addition of $CuCl_2$, 5 μl (0.13 μg) was used to measure activity of the H_2 oxidation coupled with MB reduction, and 30 μl (0.77 μg) was taken to analyze the activity of H_2 production supported by reduced MV. For the sample without Cu(II), the rate of H_2 oxidation was 7.65 nmoles H_2/min·μl sample; and the rate of H_2 production was 0.32 nmoles H_2/min·μl sample.

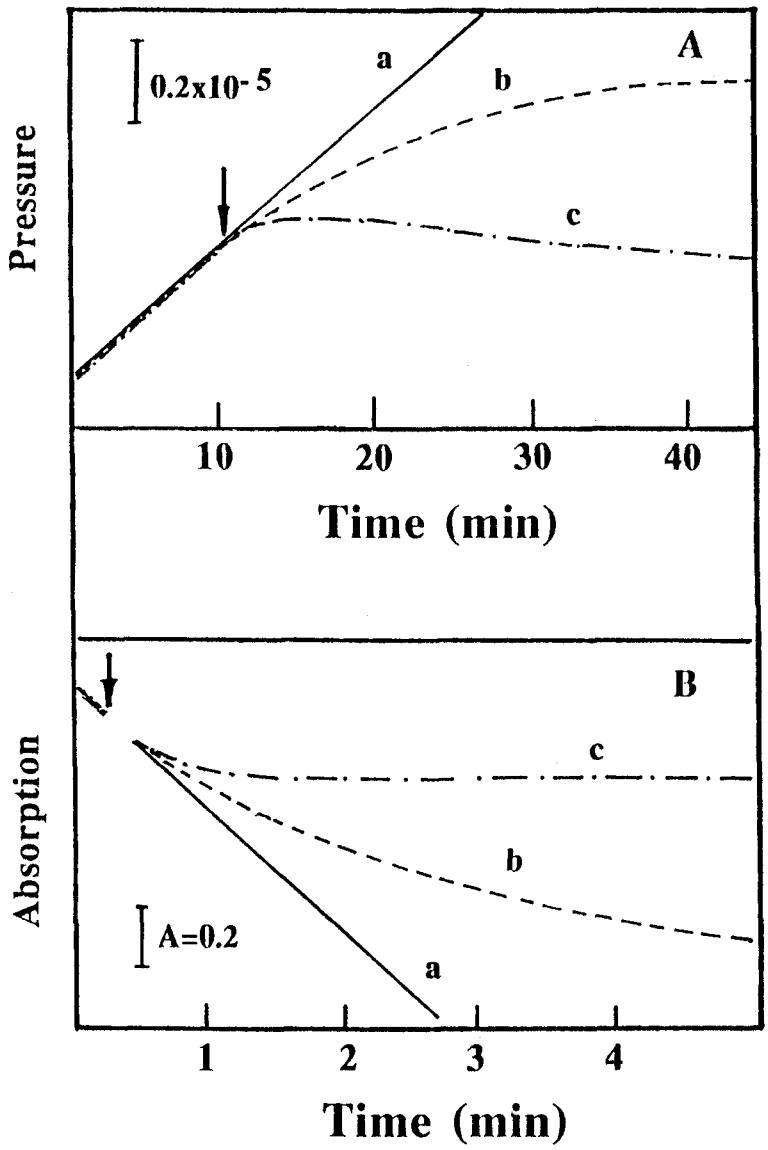

Figure IV. 1. Effects of Cu(II) on the H_2 Oxidation and D_2/H^+ Exchange Reactions Catalyzed by *A. vinelandii* Hydrogenase. In fig. A, 50 µl of hydrogenase (0.054 µg/µl) was injected at zero time into the D_2/H^+ exchange reaction mixture described in the Material & Methods. The formation of H_2 is recorded in the figure. Trace a: the formation of H_2 in the absence of Cu(II). Trace b: At time indicated by the arrow, 9.5 µM $CuCl_2$ was added. Trace c: 95 µM $CuCl_2$ was added at the time indicated by the arrow. In fig. B, 10 µl of hydrogenase (0.054 µg/µl) was injected at zero time into an H_2 oxidation/MB reduction reaction mixture. The reduction of MB was recorded in the figure. At time indicated by narrow, $CuCl_2$ was added. Trace a: instead of Cu(II), anaerobic water was injected. Trace b: 1 µM Cu(II) was added. Trace c: 10 µM Cu(II) was added.

Cu(II) Inactivation of *A. vinelandii* Hydrogenase under Turnover Condition

To prove that the Cu(II) inhibitory effect occurred at the H_2 activation step, we studied the Cu(II) inactivation under turnover conditions. When the active form of hydrogenase was introduced into the H_2 oxidation cuvette, a constant rate of methylene blue reduction was obtained (Fig. IV-1, trace 1). Following the addition of $CuCl_2$, the rate of methylene blue reduction decreased (Fig. IV-1, trace 2, &3). From these progress curves, the reduction rates were determined at 5 second intervals, and then a plot of semilog of reduction rate vs. time was made (Fig. IV-2). From these straight lines, an inactivation rate (k_{obs}) for each concentration of Cu(II) was derived. From plot of k_{obs} vs. Cu(II) concentration, a $k_{inact.}$ of 1.04×10^{-2}/s·μM (Fig. IV-2, inset) was derived.

In further experiments, the effects of substrate, methylene blue, and enzyme concentration were observed. The methylene blue was varied from 67 to 267 μM and enzyme from 1 to 12 nM. These variations did not substantially change the inactivation rate. Cu(II) inactivation under turnover conditions was irreversible. Upon addition of Cu(II) chelators (EDTA, Ally-thiourea, or thiourea) no further loss of activity was observed and the methylene blue reduction rate did not recover to the original level.

Rate and Constant of Cu(II) Inactivation of *A. vinelandii* Hydrogenase under Non-Turnover Condition

Under non-turnover conditions, the hydrogenase from *A. vinelandii* is sensitive to copper inactivation. Initially, we could not follow the time-course of the Cu(II) inactivation. When 50 μM $CuCl_2$ was added to a 0.27 μM hydrogenase solution, the inactivation to 50% residual activity occurred immediately (within 4.2 s) and then the

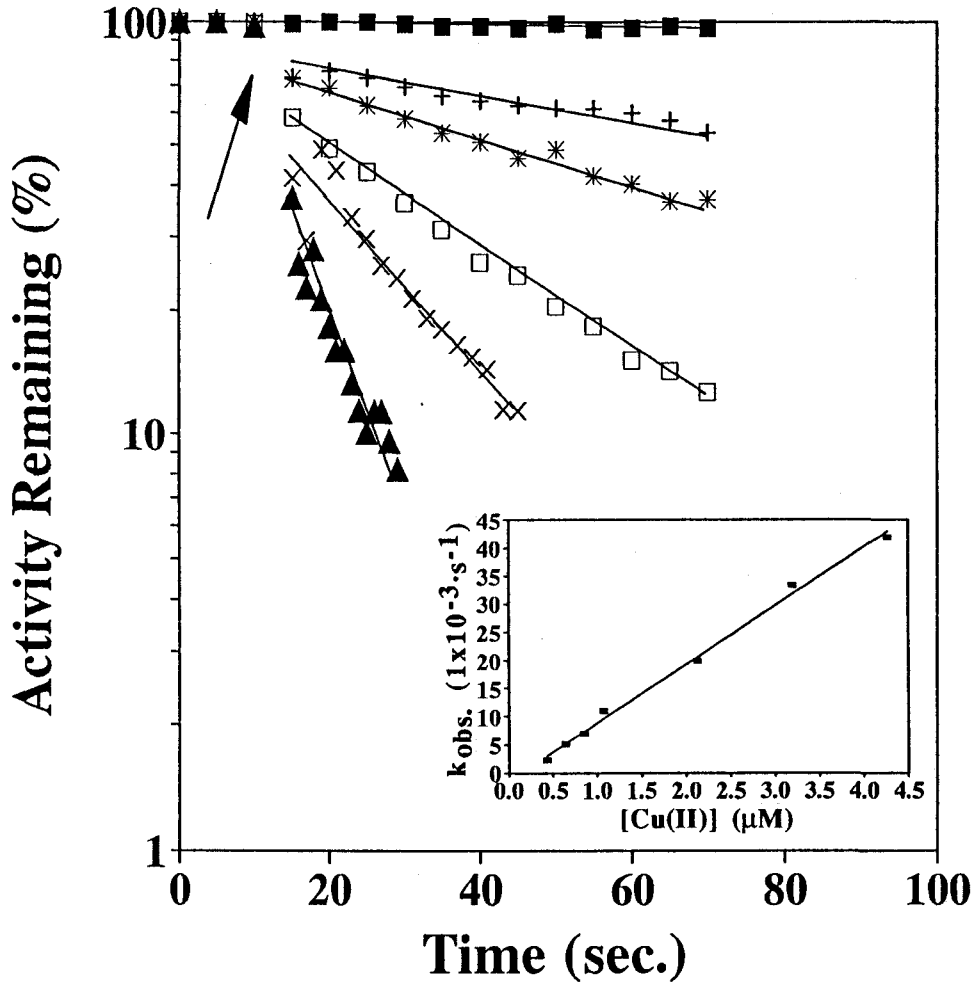

Figure IV. 2. Effect of Cu(II) Concentration on the Rate of H_2 Oxidation Inactivation under Turnover Condition. 0.4 μg of hydrogenase was injected into each of the H_2 oxidation/MB reduction mixtures at zero time. At 15 seconds, $CuCl_2$ was added to the reaction mixture: (■) 0 μM ; (+) 0.43 μM; (∗) 0.64 μM; (□) 0.85 μM; (x) 2.13 μM; (▲) 4.27 μM. The rate of MB reduction at zero time was taken as 100% activity remaining. Then at 5 second intervals, the rate of MB reduction was calculated from the progress curves as described in the text. Apparent first-order rate constants of loosing activity ($k_{obs.}$) were derived from slop of the linear line. The inset shows a plot of $k_{obs.}$ vs Cu(II) concentration.

activity remained constant. Decreasing the concentration of hydrogenase to the levels comparable to those used in turnover experiments was helpful for obtaining the time-course of hydrogenase inactivation by Cu(II). *A. vinelandii* hydrogenase (2.35 pmoles in 1 ml) was incubated with 0 to 30 μM $CuCl_2$ in the activity assay cuvette without presence of methylene blue. The time-course of loss in activity followed an apparent first-order process (Fig. IV-3). The rate for the loss of activity were derived from the data in Fig. IV-3 and plotted as $k_{obs.}$ vs Cu(II) concentration (inset in Fig. IV-3). The line is hyperbolic. To obtain an inactivation constant that is comparable with that obtained under turnover condition, the linear region from 0 to 5 μM was considered. The inactivation constant of Cu(II) inactivation of *A. vinelandii* hydrogenase under non-turnover condition was estimated to be 1.47×10^{-3}/s·μM which is 10 times less than the inactivation constant under turnover condition. At saturated concentration of Cu(II), the inactivation rate is about 1.06×10^{-2}/s.

Cu(II) Inactivation of Different Forms of *A. vinelandii* Hydrogenase

That the Cu(II) inactivation constant under turnover condition is higher than that under non-turnover condition may suggest that the occurrence of the Cu(II) inactivation required the hydrogenase to be in an active state. Three approaches were used in further experiments to test this suggestion.

Effects of Cu(II) on hydrogenase in the absence of H_2 or $Na_2S_2O_4$. To address if the Cu(II) inactivation required H_2 or another reductant, two hydrogenase samples were required--one lacking $Na_2S_2O_4$ and the other lacking H_2 and $Na_2S_2O_4$. The first sample was prepared by stripping $Na_2S_2O_4$ from isolated hydrogenase in the presence of H_2. This enzyme is referred to as E-H_2. Then, E-H_2 was degassed repeatedly to remove the H_2 and incubated E-H_2 under N_2 for 30 min. The enzyme sample is referred to as E-N_2 and contained no reductant. To obtain a sample with a

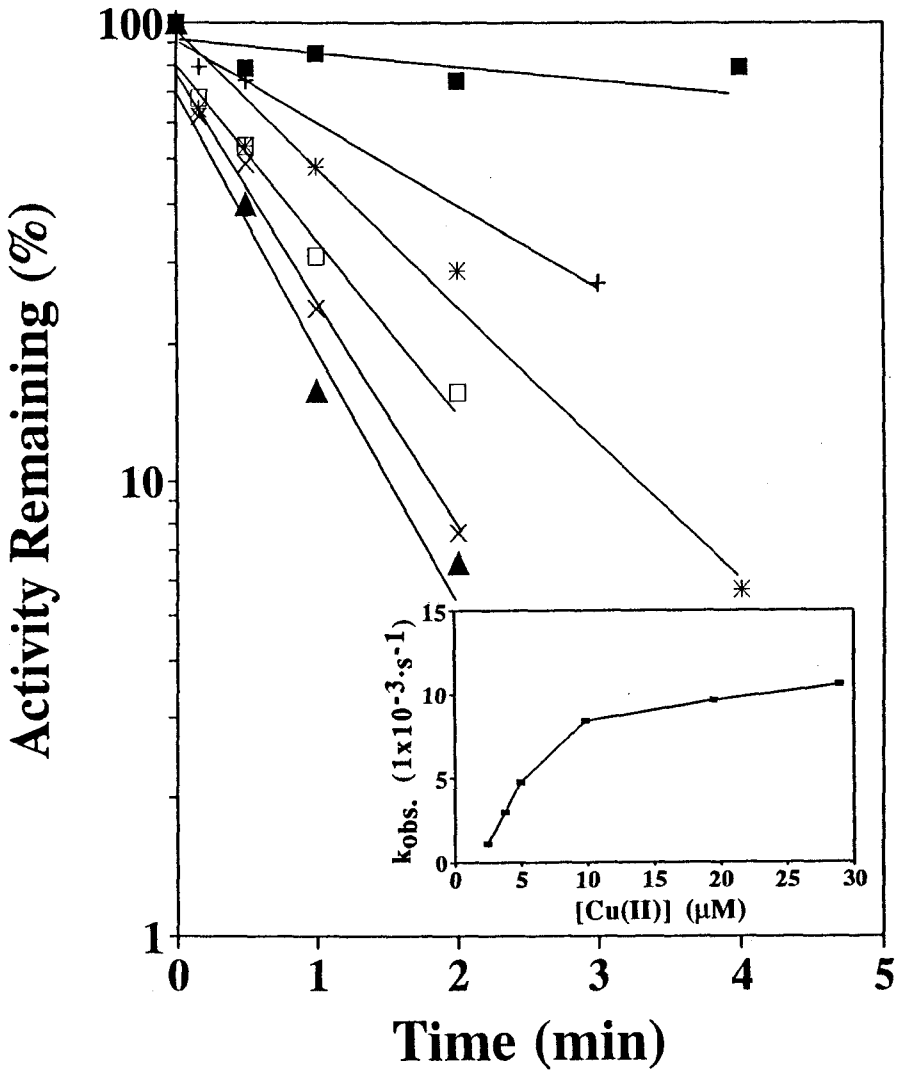

Figure IV. 3. Effect of Cu(II) Concentration on *A. vinelandii* Hydrogenase in the H_2 Oxidation under Incubation Condition. For each point, 0.23 μg of hydrogenase was incubated with following different concentrations of $CuCl_2$ in the activity assay cuvette (see Materials & Methods): (■) 2.49 μM; (+) 3.78 μM; (*) 4.97 μM; (□) 9.88 μM; (x) 19.51 μM; and (▲) 28.92 μM. At the indicated times, an H_2 saturated MB solution containing methylene blue (200 μM) and EDTA (2 mM) was injected to the cuvette to stop any further inactivation by Cu(II), and to initiate the H_2 oxidation assay. Apparent first-order rate constants for the loss of activity ($k_{obs.}$) were derived from the slops of the line. The inset shows a plot of $k_{obs.}$ vs Cu(II) concentration.

reductant other than H_2, $Na_2S_2O_4$ was added back to E-N_2 hydrogenase. The ability of Cu(II) to inactivate these different states of hydrogenase was tested with 10 μM $CuCl_2$ under either H_2 or N_2 + $Na_2S_2O_4$. The results were summarized in the Table IV-2. Both H_2-activated and $Na_2S_2O_4$-activated hydrogenase were sensitive to Cu(II) inactivation, regardless of the incubation conditions. For the E-N_2 hydrogenase, the presence of H_2 was required for the Cu(II) inactivation to occur. Apparently, the role of H_2 was to activate the catalytic site.

Table IV. 2. Effects of H_2 and $Na_2S_2O_4$ on the Cu(II) inactivation of A. *vinelandii* hydrogenase

Enzyme form	Incubation condition	Inactivation by 10 μM $CuCl_2$
E-H_2+$Na_2S_2O_4$	N_2	Yes
E-H_2	H_2	Yes
	N_2	Yes
E-N_2	H_2	Yes
	H_2+$Na_2S_2O_4$	Yes
	N_2	No
	N_2+$Na_2S_2O_4$	No
E-N_2+$Na_2S_2O_4$	N_2	Yes

Hydrogenase (0.23 μg) was incubated with 10 μM $CuCl_2$ in 1 ml of MES buffer (0.05 M, pH 6.0) in the assay cuvette as described in the Material and Methods. The Cu(II) inactivation was determined by the time course of loss in activity of H_2 oxidation. E-H_2+$Na_2S_2O_4$: the *A. vinelandii* hydrogenase solution that contained 2 mM $Na_2S_2O_4$ was evacuated and filled with H_2. E-H_2: the E-H_2 form was evacuated and fill with N_2. E-N_2+$Na_2S_2O_4$: 2 mM $Na_2S_2O_4$ was added back to the E-N_2 form of hydrogenase.

Effects of Cu(II) on the aerobically purified *A. vinelandii* hydrogenase. The aerobically purified *A. vinelandii* hydrogenase is in an unready state in term of catalytic activity. When it was introduced into activity assay solution (turnover condition), it was

activated to a certain extent, but did not obtain full activity. This means that the H_2 activation site in this enzyme is not fully activated. Full activity equal to that of the anaerobically purified *A. vinelendii* hydrogenase was only obtained following an activation period in the presence of a suitable reductants (H_2 or $Na_2S_2O_4$)(Sun and Arp, 1991). When we incubated the aerobically isolated, non-activated enzyme with 2 mM $CuCl_2$ for 4 hrs, no Cu(II) inactivation of hydrogenase was observed. However, when the enzyme was fully activated with H_2 and then 2 mM $CuCl_2$ was added, the activity of the enzyme decreased rapidly (Fig. IV-4). We also observed the effects of Cu(II) on the activation of aerobically purified *A. vinelandii* hydrogenase. At the beginning of the activation period, Cu(II) did not inactivate the enzyme. With time, the unready state of enzyme was converted to the active form as indicated by increase of activity in the control sample without Cu(II). With activation, the activity in the sample containing Cu(II) began to decrease. This suggests that the Cu(II) inactivation is related to activation of the catalytic site in *A. vinelandii* hydrogenase.

Effects of inhibitors relative to the H_2 activation site on the Cu(II) inactivation

A number of inhibitors of *A. vinelandii* hydrogenase have been characterized. Some of these inhibitors bind to the H_2 activation site of *A. vinelandii* hydrogenase. Thus, these inhibitor have been chosen in this experiment to prove if the active state of H_2 activation site is required for Cu(II) inactivation. These results have been summarized in the Table (IV-3).

(1) C_2H_2. C_2H_2 is an active-site directed, slow-binding, reversible inhibitor of *A. vinelandii* hydrogenase (Hyman and Arp, 1987a). It has been demonstrated that C_2H_2 binds to large subunit of the enzyme (Sun, et al., 1992). Because it is a reversible inhibitor, we used it to make an inactive enzyme (C_2H_2-bound hydrogenase) first, then to incubate the enzyme with Cu(II). After removal of free Cu(II), the activity of the C_2H_2-bound enzyme can be recovered to original level, in contrast to the sample not treated

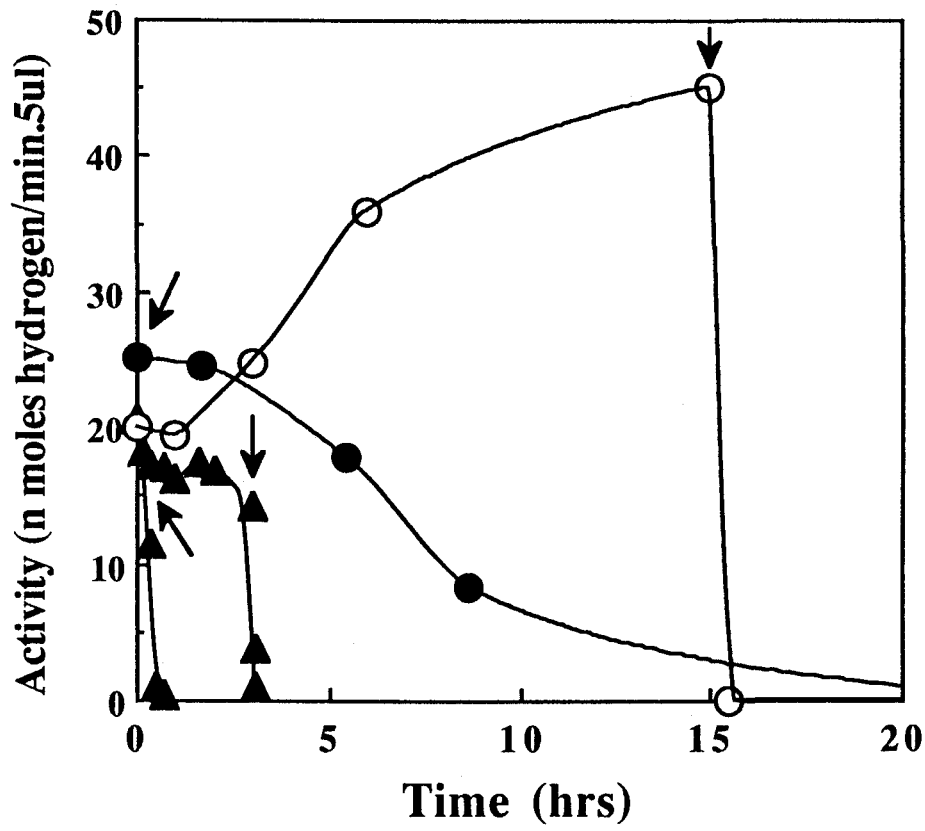

Figure IV. 4. Effects of Cu(II) on the Aerobically Purified *A. vinelandii* Hydrogenase. Aerobically purified hydrogenase (5 µl; 0.2µg/µl) was incubated with 2 mM $CuCl_2$ in 50 µl of Tris-HCl (0.02 M, pH 7.4) either under N_2 gas phase (▲), or under H_2 gas phase (●). To the enzyme sample that was incubated with Cu(II) under N_2, 2.5 mM ascorbate was injected at the arrow. Another 5 µl of aerobically purified hydrogenase (0.2 µg/µl) was activated by incubation under H_2 in the absence of Cu(II). At the end of the activation point (15.5 hrs), 2 mM $CuCl_2$ was added (○).

with C_2H_2 (Fig. IV-5). This result indicated that inhibition of the H_2 activation site prevent the access of Cu(II) to *A. vinelandii* hydrogenase.

(2) CO. CO is another active-site directed, reversible inhibitor. EPR spectroscopy showed that ^{13}CO affected the EPR signals which arose from Ni(Van der Zwaan, et al., 1990). CO, like H_2, was able to compete with C_2H_2 (Hyman and Arp, 1987a), thus the CO was presumed to bind mutually exclusively with H_2 at the normal H_2 binding site. However, CO is a fast binding inhibitor. Therefore, we directly incubated the *A. vinelandii* hydrogenase with Cu(II) in the presence of 101 kPa CO. Almost no Cu(II) inactivation was observed in the enzyme sample with CO, indicating that the fast equilibrium between CO and H_2 binding site is able to prevent the enzyme from the Cu(II) inactivation.

(3) O_2. O_2 is a reversible inhibitor and an irreversible inactivator of *A. vinelandii* hydrogenase. In the presence of H_2, O_2 inhibits the *A. vinelandii* hydrogenase reversibly, otherwise irreversibly without presence of H_2. In this experiment, we treated *A. vinelandii* hydrogenase with O_2 in the presence of 80% H_2. Under this condition, the H_2 binding site is presumed to be protected by H_2 (Seefeldt and Arp, 1989). The UV-vis spectroscopy indicated that [Fe-S] cluster has been oxidized (Chapter III). The effect of $^{17}O_2$ on the EPR of nickel in hydrogenase of *Chromatium vinosum* indicated that the O_2 can tightly bind in the vicinity of the Ni (Van der Zwaan, et al., 1990). When O_2 and H_2 treated *A. vinelandii* hydrogenase was chosen to test the Cu(II) inactivation, the result showed that it required more Cu(II) (9.54 μM) to get 50% inactivation relative to untreated enzyme (4 μM). This partial protective effect on the enzyme from the Cu(II) inactivation suggested that the inactive state of another site close to the Ni center in the enzyme could effect the Cu(II) inactivation.

(4) Metal. *A. vinelandii* hydrogenase is a Ni and Fe containing enzyme. Ni has been strongly suggested to be the H_2 activation site in this category of hydrogenase.

Table IV. 3. Effects of Factors Relative to H_2 Activation Site on the Cu(II) Inactivation in *A. vinelandii* Hydrogenase

Factors	Turnover condition		Non-turnover condition	
	Inhibitory effect on enzyme	Inhibitory effect on Cu(II) inactivation	Inhibitory effect on enzyme	Inhibitory effect on Cu(II) inactivation
C_2H_2	--	--	+	+ (100%)[a]
CO 100%	+	--	-	+ (95%)[b]
O_2(+H_2) 20%	--	--	+	+ (partial)[c]
DTT 2 mM	-	+ (100%)[d]	-	+ (100%)[d]
Ni(II)	+ 1/2[mM][e] 4.74	+ (80%) [f] at 8 mM Ni	-	+(partial)[c] at 1-2 mM Ni
Fe(II) 4 mM	-	-	--	--
Fe(III) 0.01-0.1 mM	+ 1/2[mM][e] 0.046	-	-	-
Co(II) 0.5-2 mM	-	-	-	-
Zn(II)	+ 70% at 4 mM[g]	-	+ 1/2[mM][e] 4.95	-

+: The factor inhibits catalysis or inhibits inactivation. -: The factor does not inhibit catalysis or inactivation. [a]: The enzyme recovered for activity, even if it was incubated with Cu(II). [b]: 2.53 μg hydrogenase was incubated with 101 kPa CO and 12 μM Cu(II) in 50 μl MES buffer (0.05 M, pH 6.0). At 10 min, 95% of activity remained compared with the enzyme treated with only 101 kPa CO. At the same time, the Cu(II) treated hydrogenase has 30% of activity remained compared with the non-Cu(II) treated hydrogenase. [c]: See text. In these samples, the Cu(II) inactivation was still observed, but it required a higher concentration of Cu(II) to get the same extent of inactivation. [d]: In the presence of 2 mM DTT, no Cu(II) inactivation was observed. [e]: At these concentration (mM) of inhibitor, 50% of activity was inhibited. [f]: The rate of the Cu(II) inactivation decreased to 20% of that without presence of Ni (II). [g]: At 4 mM Zn(II), the hydrogenase has 70% of activity remaining.

Fe has been demonstrated to be present in [Fe-S] clusters that are presumed to be electron transfer mediators. Given these knowledge, we tested the effects of Ni and Fe on the *A. vinelandii* hydrogenase under turnover and non-turnover conditions, and the effects of Ni and Fe on the Cu(II) inactivation. The Ni(II), Fe(III) and Zn(II) all have an inhibitory effect on the enzyme under turnover condition (Table IV-II). But only Ni(II) has protective effect on the enzyme from the Cu(II) inactivation. In the presence of 8 mM of Ni(II) under turnover condition, the inactivation rate of the Cu(II) inactivation decreased from $1.12x10^{-2}$/s (without Ni(II)) to $0.29x10^{-2}$/s. Under non-turnover conditions, only Zn(II) was still able to inhibit the activity. But Zn(II) did not protect the enzyme from the Cu(II) inactivation. Although Ni(II) did not inhibit the activity of *A. vinelandii* hydrogenase under non-turnover conditions, treatment of the enzyme with 1 mM of Ni(II) raised the Cu(II) concentration from 7.56 to 11.30 for obtaining half inactivation of the enzyme activity (1/2[μM]). An unexplainable result is that, unlike the situation observed under turnover condition, higher concentration of Ni (II) is unable to prevent enzyme from the Cu(II) inactivation. In several experiments, we found the optimal concentration of Ni for protection was between 1 and 2 mM. Only Ni(II) among the metals that we tested has the ability to protect enzyme from the Cu(II) inactivation, although other metals could be inhibitors of turnover reaction. These results suggested that the active state of the H_2 activation site that is relative to the Ni center was required for the occurrence of the Cu(II) inactivation in the *A. vinelandii* hydrogenase.

Optical Properties of Cu(II) Inactivated *A. vinelandii* Hydrogenase

Addition of 0.25 mM Cu(II) to ferredoxin I from *Desulfovibrio africanus* produced bleaching of the [4Fe-4S] chromophore. The same effects was observed in the hydrogenase from *D. gigas* (Fernandez, et al., 1989). It has been proposed that Cu(II) inactivation affects the electron transfer step from H_2 activation to the electron acceptors.

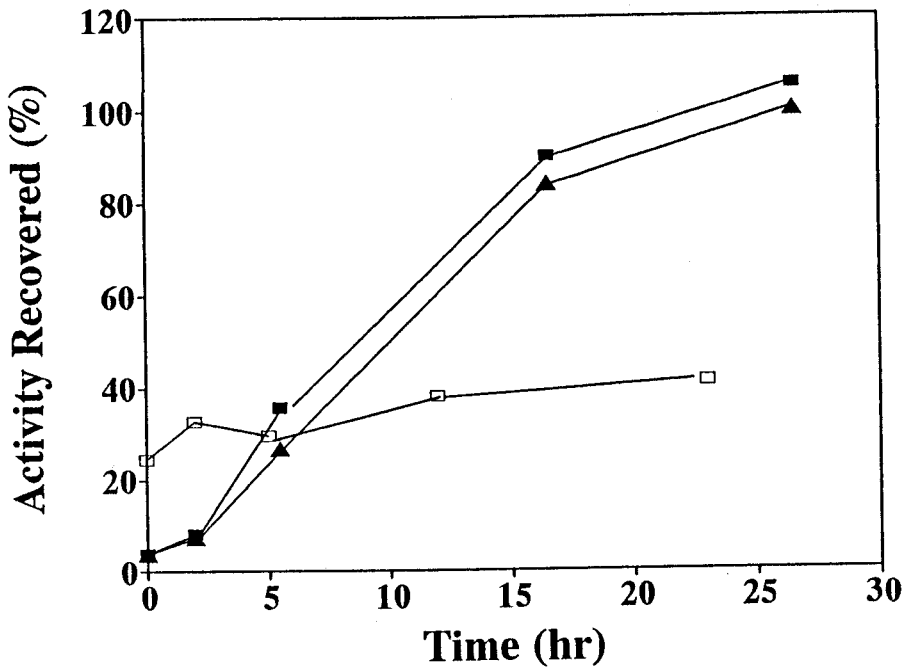

Figure IV. 5. Effects of Cu(II) on the C_2H_2 Inhibited *A. vinelandii* Hydrogenase. Hydrogenase (17.2 μg) in 150 μl of Tris-HCl (0.02 M, pH 7.4) was incubated with 101 kPa C_2H_2 for 12 hrs. At the end of incubation, 95% of activity of enzyme was inhibited. This C_2H_2 inhibited hydrogenase was divided into two parts. One part was evacuated repeatedly and flushed with N_2 to remove the free C_2H_2, then incubated with 0.5 mM $CuCl_2$ for 40 min (▲). Another part remained under 101 kPa C_2H_2 and was not incubated with $CuCl_2$ (■). Additional hydrogenase (8.6 μg) in 75 μl of Tris-HCl (0.02 M, pH 7.4) was used as a control sample (□), and incubated with 0.5 mM $CuCl_2$. At 40 min after incubation, 58% of activity of enzyme lost. Then, to remove the free Cu(II), all three samples were passed through a Sephodex G-25 column (1x10 cm) that was equilibrated and developed with Tris-HCl (0.02 M , pH 7.4) containing 2 mM EDTA under H_2 gas. All fractions containing protein were pooled and transferred to the recovery vials. The Fig. shows the recovery results. The initial activity was taken as 100%.

Consistent with this model, the [Fe-S] cluster was destroyed during the Cu(II) inactivation. Therefore, we determined the UV-vis absorption spectrum of the Cu(II) inactivated *A. vinelandii* hydrogenase. Incubation of hydrogenase with 50 μM $CuCl_2$ modified the optical absorption spectrum. During the inactivation process, a bleaching of the absorption arising from [Fe-S] clusters (at 420 nm) was observed (Fig. IV-6).

In addition to the bleaching of [Fe-S] clusters, new absorption bands were observed at 320 and 300 nm in the difference spectrum of the Cu(II) treated enzyme against isolated, non-Cu(II) treated enzyme. These absorptions corresponded with inactivation of the activity in a time course curve (Fig. IV-7). When BSA was treated with the same concentration of Cu(II), no such absorption hands were observed, indicative of specificity of these absorptions to hydrogenase. Although Cu(II) is a colored transition metal ion, free Cu(II) ions in solution are not optically active. However, Cu(II) becomes optically active when bound to a protein (Tieghem, et al., 1991) due to d-d transitions which give rise to absorption in the 400-800 nm region. In hydrogenase and BSA, this absorption is located at 640 nm. Ligand to metal charge-transfer (LMCT) will occur in the 250-400 nm region. The wavelength characteristics of the charge transfer absorption depend on the nature of the ligands coordinated to the Cu(II) ion. Extensive studies on the charge transfer absorption of Cu(II)-imidazole chromophores indicated these chromophores exhibit three type of LMCT absorption (Fawcett, et al., 1980). The first one originates from the σ-symmetry nitrogen donor lone pair and the other two from π-symmetry ring orbitals, one with mostly carbon character (π_1) and the other with mostly nitrogen character (π_2). These LMCT absorptions occur at about 220, 330, and 260 nm, respectively (Tieghem, et al., 1991). In addition to the imidazole ring, the thiol group in the enzyme easily ligates to Cu(II) or Cu(I). The absorption of thiole-Cu(II) complexes occur at 230 nm (Khan and Sorenson, 1991). Although, the Cu(II) inactivated *A. vinelandii* hydrogenase exhibited the absorption at 242 nm, this absorption was not specific to the hydrogenase and to the Cu(II)

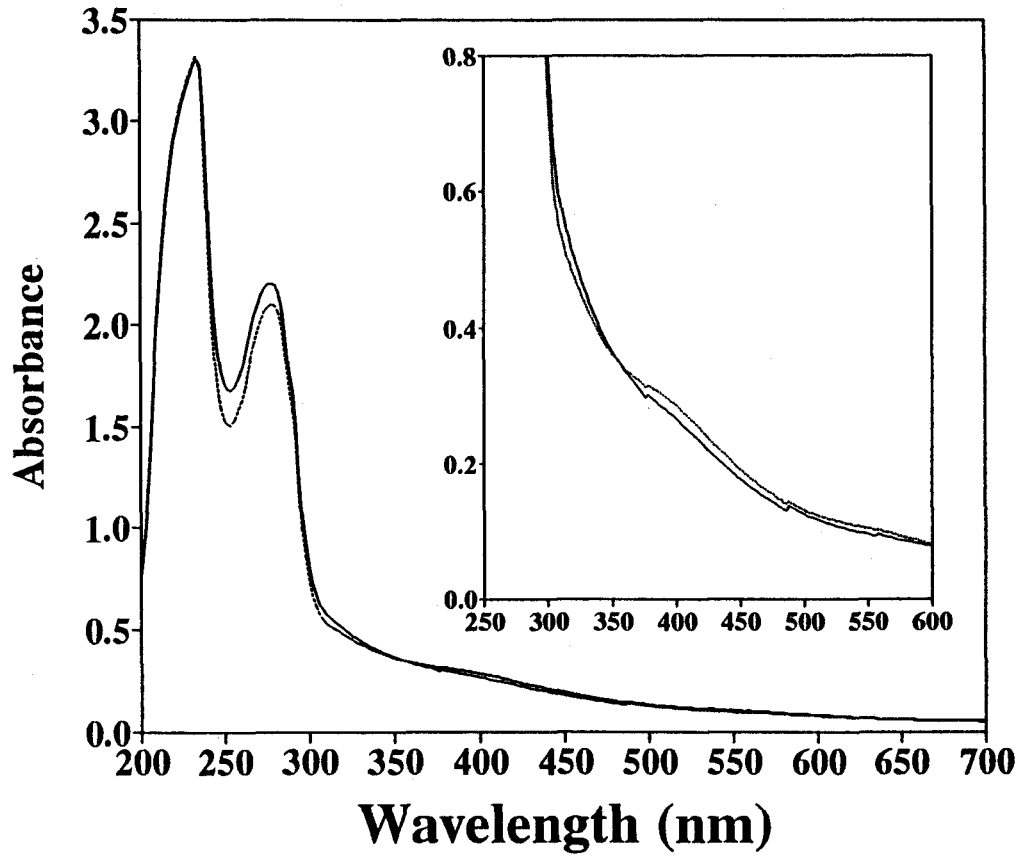

Figure IV. 6. Effect of Cu(II) on UV-Vis. Spectra of *A. vinelandii* Hydrogenase. Hydrogenase was first stripped of dithionite. Then, 101 μg hydrogenase in 480 μl of Tris-HCl (0.02 M, pH 7.4) was transferred into a H_2 filled cuvette (1 cm light path) and a scan from 700 nm to 200 nm was recorded (-----). Then 50 μM $CuCl_2$ was added to the enzyme sample. At 85 min after addition of Cu(II) when 65% of enzyme was inactivated, the scan from 700 nm to 200 nm was recorded again (——).

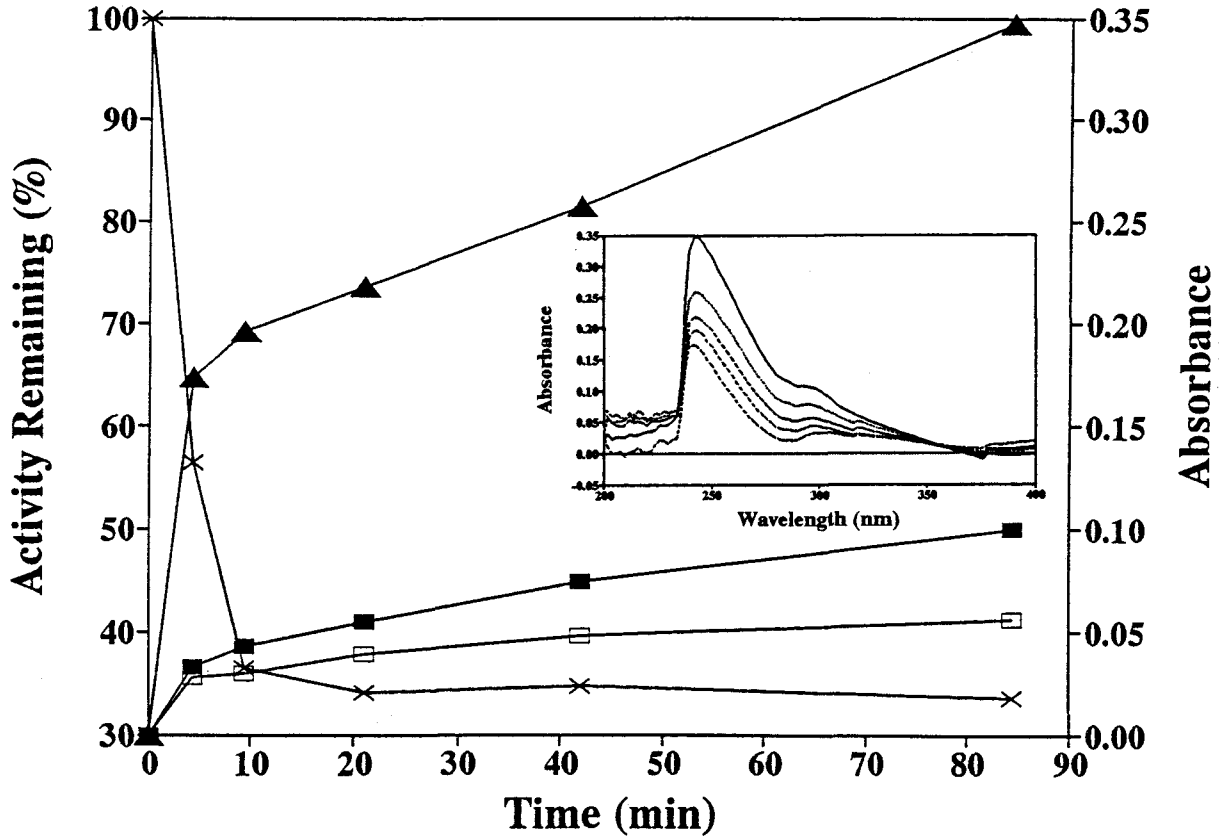

Figure IV. 7. Relationship between loss of H_2 oxidation and changes of absorption upon incubation of *A. vinelandii* hydrogenase with Cu(II). Hydrogenase was first stripped of dithionite. Then, 101 μg hydrogenase in 480 μl of Tris-HCl (0.02 M, pH 7.4) was transferred into a H_2 filled cuvette (1 cm light path), and used as background. After injection of 50 μM $CuCl_2$, the scans from 700 nm to 200 nm were recorded at indicated times. Meanwhile, 1 μl samples were withdraw for determination of enzyme activity. The absorption changes that corresponded to the activity loss (x) at 242 nm(▲), 300 nm (■), and 320 nm (□) were taken from scan spectra. Inset shows the UV-vis difference spectrum of Cu(II) inactivated hydrogenase minus spectrum of hydrogenase incubated in the absence of Cu(II).

inactivation, because a similar absorption occurred in the BSA control. Furthermore, the appearance of the 242 nm absorption in hydrogenase did not correspond to the loss of activity. Given this knowledge, the Cu(II) inactivation seemed to be related to the formation of Cu(II)-imidazole complexes, in addition to the destruction of [Fe-S] clusters.

Discussion

Cu(II) inactivate hydrogenase activity. Given the general interaction between the protein and Cu(II), it is not surprising to find this Cu(II) inactivation in hydrogenase. Amide hydrogens of protein are easily substituted by Cu(II) ion with the formation of stable 5- or 6-membered chelate rings. The imidazole of histidine and the sulhydryl group of cysteine residues are also effective ligands for binding of Cu(II) to the protein (Sigel and Martin, 1982). The surprise is that hydrogenases from different organisms exhibited different sensitivity to Cu(II) inactivation, and that even in the same hydrogenase, different kinds of reactions were inhibited by different concentration of Cu(II). The hydrogenase from the green sulfur bacterium *Chlorobium limicola* was only slight inhibited by 0.5 mM Cu(II) in the H_2 oxidation assay (Serebryakova, et al., 1987). The purified hydrogenase from *D. gigas* could be inhibited by 0.5 mM Cu(II) in terms of H_2 oxidation and isotope exchange reaction. However, the H_2-oxidation reaction is much more sensitive to the Cu(II) inactivation than the isotope exchange reaction. In this paper, we characterized the Cu(II) inactivation in the *A. vinelandii* hydrogenase. Two interesting points are worthy of mention. First, the purified *A. vinelandii* hydrogenase is extremely sensitive to the Cu(II) inactivation. 1 to 30 μM Cu(II) could inactivate the H_2 oxidation activity to less than 10% in a few minutes. Second, under turnover conditions the Cu(II) inactivated both the H_2 oxidation and isotope exchange reactions (Table IV-1, Fig. IV-1).

The inhibition of isotope exchange by Cu(II) suggests that the Cu(II) affect the H_2 activation step of the catalytic reaction of hydrogenase. In addition, the Cu(II) could affect the electron transfer from the H_2 activation site to the electron acceptor. For the *D. gigas* hydrogenase, Cu(II) inhibited H_2 oxidation more rapidly than the isotope exchange reaction. Therefore, it was suggested that the Cu(II) affected the electron transfer step, rather than H_2 activation (Fernandez, et al., 1989). We carefully compared the Cu(II)

inactivation of both the H_2 production and H_2 oxidation reactions. The same concentration of Cu(II) gave about the same extent of inactivation of both reactions (Table IV-1). For the comparison between isotope exchange and H_2 oxidation reactions in term of the Cu(II) inhibitory effect, samples from the same batch of *A. vinelandii* hydrogenase were examined under turnover condition. Both reactions are sensitive to the Cu(II) inactivation (Fig. IV-1). Therefore, we believe that in *A. vinelandii* hydrogenase Cu(II) affects the H_2 activation step. Further observations indicated that the H_2 activation site should be in active state for the Cu(II) inactivation to occur. The presence of H_2 is required (Table IV-2), while the other substrate does not affect the Cu(II) inactivation rate. The inactivation constant of the Cu(II) inactivation under turnover condition is 10 times higher than that under non-turnover condition. The inhibits which bind to the H_2 activation site prevented the Cu(II) inactivation (Table IV-3). The aerobically purified hydrogenase where the H_2 activation site was not fully activated was not sensitive to the Cu(II) inactivation unless the sample was activated (Fig. IV-4).

Why does the occurrence of Cu(II) inactivation require the catalytically active form of hydrogenase? A similar story was described in the inhibition of *A. vinelandii* hydrogenase by C_2H_2 (Hyman and Arp, 1987a). C_2H_2 was demonstrated to be a slow and tightly binding inhibitor to the H_2 activation site of *A. vinelandii* hydrogenase. Thus, the active state of the H_2 activation is required for the C_2H_2 binding. In the case of Cu(II), we propose two alternative explanations for the requirement of an active H_2 activation site in the Cu(II) inactivation. First, the active form of *A. vinelandii* hydrogenase has an active H_2 activation site that is accessible to the Cu(II) ions. Second, the real inhibitor could be Cu(I), thus the active H_2 activation site is required for the reduction of Cu(II) to Cu(I). Because the lifetime of Cu(I) is very short (< s) in the water solution (Cotton and Wilkinson, 1976)., we did not attempt to prove that the real inhibitor in the Cu(II) inactivation was Cu(I). Nonetheless, some results could be explained by using this proposal. Under non-turnover condition, when non-diluted hydrogenase (0.2-

0.3 μM) was used to test the Cu(II) inactivation, the inactivation occurred in seconds, and then no further inactivation was observed. It seems that initially the Cu(I) was rapidly produced by higher concentration of enzyme, and consequently the activity of enzyme decreased rapidly. The oxidized H_2 activation site could not reduce any additional Cu(II), no further inactivation was observed.

Regardless of what the real inhibitor is, the hyperbolic relationship between $k_{abs.}$ and Cu(II) concentration under non-turnover condition indicated that the inactivation is a saturable process (Fig. IV-3). Thus the following kinetic mechanism can be used for these two proposed explanations for the Cu(II) inactivation.

$$E^* + I \underset{k_2}{\overset{k_1}{<====>}} E^*I \underset{k_4}{\overset{k_3}{<=====>}} EI'$$

An initial rapid conversion of the active hydrogenase (E*) to a transient E*I complex is followed by a slow conversion of E*I to an inactivated EI' complex. In this process, the E* could be oxidized or be inactivated to E, and the conversion of I to I' could involve a reduction of I. Because the Cu(II) inactivation is irreversible, the reverse rate constants k_4 and k_2 must be much smaller compared with the forward rate k_3 and k_1. Because the step from E*I to EI' is the rate limiting step, k_3 can be estimated from the inactivation rate at the saturable concentration of inhibitor (assuming k_4=0). In this case, k_3 is about 1.06×10^{-2}/s. This value is much lower than the turnover number of *A. vinelandii* hydrogenase (2000/s). Under turnover condition, the inactivation constant increased 10 times (Fig. IV-2). This increasing in inactivation constant could be due to the increase in the association constant between E* and I, because under turnover condition the H_2 activation site remained in the active state.

During the Cu(II) inactivation, the absorption arising from the [Fe-S] clusters was bleached (Fig. IV-6). The bleaching of the [Fe-S] absorption could be due to reduction of

these clusters, if Cu(II) itself is acting as an electron mediator. However, the re-oxidation of [Fe-S] could not occur after the Cu(II) inactivation, and loss of Fe has been found in the Cu-inactivated enzyme (Fernandez, et al., 1989). Therefore, the bleaching of the [Fe-S] absorption was not due to the reduction of the [Fe-S] clusters, but most probably to the destruction of these clusters. In the *D. gigas* hydrogenase, the destruction of the [Fe-S] clusters during the Cu(II) inactivation implied that the [Fe-S] clusters was a component of electron transfer, rather than the component of H_2 activation site, because destruction did not effect the isotope exchange reaction. However, in the *A. vinelandii* hydrogenase, the situation is not so simple. Cu(II) apparently destroyed the [Fe-S] clusters, and also inhibited the isotope exchange reaction. We proved that the active state of the H_2 activation site was required for the Cu(II) inactivation. Obviously, the Cu(II) inactivation was related to the H_2 activation site in someway. If the Cu(II) inactivation destroyed the H_2 activation site, does that mean the [Fe-S] cluster is a component of H_2 activation site? Perhaps the Cu(II) destroys the H_2 activation site and the [Fe-S] cluster in the same process. But we have no experimental results to suggest or refute this possibility, i.e. the [Fe-S] cluster might be the component of the H_2 activation site in *A. vinelandii* hydrogenase, but not in the *D. gigas* hydrogenase. Three notable differences have been found to exist between the *A. vinelandii* hydrogenase and the *D. gigas* hydrogenase. First, *D. gigas* hydrogenase catalyzes H_2 evolution as rapidly as H_2 oxidation, while *A. vinelandii* and *B. japonicum* hydrogenases catalyze H_2 oxidation efficiently (low Km and high Vmax), but only slowly evolve H_2 (Arp, 1985, Hatchikian, et al., 1978, Seefeldt and Arp, 1986). Second, unlike the *D. gigas* hydrogenase, the *A. vinelandii* hydrogenase exhibited a very weak Ni signals in EPR spectroscopy(Seefeldt, 1989). EPR signals arising from [Fe-S] clusters could be detected, but they were distinct from those observed in *D. gigas* hyrogenase where no typical g=1.94 signal appeared. Using the oxidized *A. vinelandii* hydrogenase, Seefeldt observed complex EPR signals which were assigned to interactions between [Fe-S] cluster and Ni center in this enzyme

(Seefeldt, 1989). Therefore, these differences in the EPR signals suggest that the H_2 activation site in the *A. vinelandii* hydrogenase might be different from that of *D. gigas* hydrogenase. But unfortunately, we do not yet have EXAFS data from the *A. vinelandii* hydrogenase or functionally related hydrogenase from *A. eutrophus* or *B. japonicum* to make comparison with the *D. gigas* hydrogenase. In the *D. gigas* hydrogenase, EXAFS studies indicated that no Fe existed around the Ni center(Scott, et al., 1984). Third, the primary structure of *A. vinelandii* hydrogenase has been shown to be different from that of *D. gigas* hydrogenase, except with conserved regions for all Ni-containing hydrogenases (see Chapter 1). This discrepancy suggests that the coordinating environment of the H_2 activation site in the *A. vinelandii* hydrogenase must be different from that in the *D. gigas* hydrogenase. These three differences implied that the mechanism of H_2 activation in *A. vinelandii* hydrogenase could be different from that in the *D. gigas* hydrogenase. Therefore, we suspected that the H_2 activation site of *A. vinelandii* hydrogenase involved the [Fe-S] component.

References

Adams, M. W. W. (1990) *Biochim. Biophys. Acta 1020*, 115-145.

Arp, D. J. (1985) *Arch. Biochem. Biophys. 237*, 504-512.

Cotton, F. A. & Wilkinson, G. (1976) *New York* 412-413.

Cypionka, H. & Dilling, W. (1986) *FEMS Microbiol. Lett. 36*, 257-260.

Fawcett, T. G., Bernarducci, E. E., Krogh-Jespersen, K. & Schugar, H. J. (1980) *J. Am. Chem. Soc. 102*, 2598-2604.

Fernandez, V. M., Rua, M. L., Reyes, P., Cammack, R. & Hatchikian, E. C. (1989) *Eur. J. Biochem. 185*, 449-454.

Hatchikian, E. C., Bruschi, M. & LeGall, J. (1978) *Biochem. Biophys. Res. Commun. 914*, 299-303.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1987a) *Biochemistry 26*, 6447-6454.

Hyman, M. R. & Arp, D. J. (1987b) *Appl. Environ. Microbiol. 53*, 298-303.

Khan, M. F. & Sorenson, J. R. (1991) *Journal of Inorganic Biochemistry 41*, 221-234.

Maroney, M. J., Copas, G. J. & Bagyinka, C. (1990) *J. Am. Chem. Soc. 112*, 7007-7068.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1992) *FEMS Microbiology Reviews 88*, 109-135.

Schlegel, H. G. (1954) *Flora 141*, 1-15.

Scott, R. A., Wallin, S. A., Czechowski, M., Der Vartanian, D. V., Le Gall, J., Peck, H. D. J. & Moura, I. (1984) *J. Am. Chem. Soc. 106*, 6864-6865.

Seefeldt, L. C. (1989) Ph. D thesis, University of California, Riverside.

Seefeldt, L. C. & Arp, D. J. (1989) *Biochemistry 28*, 1588-1596.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Serebryakova, L. T., Zorin, N. A. & Gogotov, I. N. (1987) *Biochemistry Engl. Transl. Biokhimiya 52*, 779-785.

Sigel, H. & Martin, R. B. (1982) *Chem. Rev. 82*, 385-426.

Sun, J.-H. & Arp, D. J. (1991) *Arch. Biochem. Biophys. 287*, 225-233.

Sun, J. H., Hyman, M. R. & Arp, D. J. (1992) *Biochemistry 31*, 3158-3165.

Tieghem, E., Van Dael, H. & Van Cauwelaert, F. (1991) *Journal of Inorganic Biochemistry 42*, 119-131.

Van der Zwaan, J. W., Coremans, J. M. C. C., Bouwens, E. C. M. & Albracht, S. P. J. (1990) *Biochim. Biophys. Acta. 1041*, 101-110.

CHAPTER V.

SUMMARY

Hydrogenase activity in *Azotobacter vinelandii* was first described by Phelps and Wilson (Phelps and Wilson, 1941). Hyndman et al (Hyndman, et al., 1953) characterized several properties of this enzyme in cell-free extracts, establishing that the enzyme was membrane-bound. In 1984, the *A. vinelandii* hydrogenase was partially purified by Kow and Burris in the single subunit form with 60 kDa size (Kow and Burris, 1984). In 1986, this hydrogenase was demonstrated to be a heterodimer composed of subunits of 67 and 31 kDa and to contain 6.6 mol Fe and 0.68 mol Ni per mol hydrogenase (Seefeldt and Arp, 1986). Compared with a number of Ni-containing hydrogenases from sulfate reducing bacteria, the Ni content of *A. vinelandii* hydrogenase is lower. Chapter I of this dissertation discussed the Ni content in *A. vinelandii* hydrogenase. Both Biuret and Lowery methods for protein determination overestimate the protein content of *A. vinelandii* hydrogenase by 1.91(±0.32) on the basis of amino acid composition analysis. After correction for the overestimate of protein concentration, the Ni content is raised to 1.30 mol per mol hydrogenase. The Fe exists in the [Fe-S] clusters in the hydrogenase. The EPR spectrum of the reduced and active *A. vinelandii* hydrogenase exhibited a complex "g=1.94" type signal typical of an 4Fe-4S cluster interacting with another paramagnetis species. Upon oxidation by O_2, the EPR signal of "g=1.94" disappeared and a new signal with g=2.02 appeared, indicating the presence of [3Fe-4S] cluster (Seefeldt, 1989). Consistant with these observations, the UV-vis spectrum of the *A. vinelandii* hydrogenase presented in Chapter III indicated the presence of [Fe-S] clusters. A broad absorption band in the range of 300-600 nm increases in intensity upon oxidation of the protein. Given that the ratio of Fe to Ni is 11, the *A. vinelandii* hydrogenase contains at least one [3Fe-4S] and two [4Fe-4S] clusters. Given that the small subunit

contains 10 conserved cysteinyl residues in contrast to only 5 in the large subunit, it seems apparent that some of [Fe-S] clusters must be located in the small subunit (Menon, et al., 1990).

Inhibitors can often be helpful in elucidating the function of the subunits and the metal clusters or centers of hydrogenase in catalysis. A variety of inhibitors of hydrogenase activity have been examined kinetically, included C_2H_2, O_2, HCN, NO, and CO. In the works presented in this dissertation, the value of these inhibitors has been further explored.

Proteins with site-specific substitution of conserved cysteines in the small subuint (HoxK) have no or greatly decreased activities (Sayavedra-Soto and Arp, 1993), indicating that small subunit was essential for the activity of *A. vinelandii* hydrogenase. As to the large subunit, it is likely to bind the Ni (Przybyla, et al., 1992). The Ni has been strongly suggested to be the site of H_2 activation for the Ni-containing hydrogenases (Przybyla, et al., 1992). The works presented in Chapter II of this dissertation provided the first biochemical evidence that demonstrated the H_2 binding site is located in the large subunit of *A. vinelandii* hydrogenase. C_2H_2 is a slow-binding inhibitor of *A. vinelandii* hydrogenase. It is competitive vs. H_2, indicating that C_2H_2 binds to the H_2-binding site of the enzyme (Hyman and Arp, 1987). The works of Chapter II show that $^{14}C_2H_2$ binds tightly to only the large subunit of *A. vinelandii* hydrogenase. During binding, no transformation of C_2H_2 to another chemical compound was detected. Unfortunately, proteolysis leads to de-stabilization of ^{14}C-label. This disappointing result prevented us from identifying a specific amino acid residue to which the C_2H_2 was bound (see Appendix). Nonetheless, specific binding of $^{14}C_2H_2$ elucidated the location of the H_2 binding site in the large subunit.

The nature of the [Fe-S] clusters of hydrogenases have been examined. Mossbauer spectra analysis indicated that the [3Fe-4S] cluster in *D. gigas* hydrogenase

was magnetically isolated from the Ni paramagnetic center although the [3Fe-4S] cluster could interact with the reduced [4Fe-4S] clusters in this hydrogenase. This is consistent with the EXAFS data that indicate that no Fe is near the Ni (see Chapter I). The nature of [Fe-S] clusters in the *A. vinelandii* hydrogenase could be different from that in the *D. gigas* hydrogenase, because the typical "g=1.94" type EPR signal of the $[4Fe\text{-}4S]^{+1}$ cluster exists in the *A. vinelandii* hydrogenase (Seefeldt, 1989), but not in the *D. gigas* hydrogenase (Teixeira, et al., 1990, Teixeira, et al., 1989). With regard to the function of the [Fe-S] clusters in catalysis, little is known. These clusters were suggested to be electron mediators from the H_2 activation site to the electron acceptors. The work presented in the Chapter III examined the effects of substrate and inhibitor on the absorption spectra of [Fe-S] clusters. The results indicated that inhibitors which bind reversibly to the H_2 activation site do not affect the nature of the [Fe-S] clusters, but inhibitors which bind irreversibly to hydrogenase could prevent the reduction of the [Fe-S] clusters by H_2. Binding of the substrate, H_2, and the inhibitor, C_2H_2, do not alter the absorption intensity associated with [Fe-S] clusters. The oxidation and reduction of the [Fe-S] clusters in either the H_2 or C_2H_2 bound enzyme are still able to occur upon addition or removal of O_2. In contrast to this, the absorption change due to oxidized [Fe-S] clusters which occurred upon binding of CN^- to *A. vinelandii* hydrogenase did not decrease upon incubation with H_2, but did decrease upon incubation with dithionite. This observation indicated that the reduction of [Fe-S] clusters depended upon the other functional centers (maybe the H_2 activation center) during H_2 oxidation.

It was also observed that NO and Cu(II) destroyed the [Fe-S] clusters (Chapter III & IV). Because these inhibitors inactivate the isotope exchange activity of *A. vinelandii* hydrogenase (Hyman and Arp, 1991) & Chapter IV), they were proposed to interact with H_2 binding site. These observations raised a question, i. e. does the H_2 binding center involve an [Fe-S] cluster component, or do these inhibitors attack the enzyme at multiple sites?

The Ni center has been suggested to be the H_2 binding site on the basis of paramagnetic spectroscopy studies (see Chapter I). Ni signals in the EPR spectrum of the *D. gigas* hydrogenase showed three forms: Ni-A; Ni-B; and Ni-C. Ni-A and Ni-B represented inactive states of enzyme. In these states of the enzyme, the Ni center is inaccessible to solvent H^+. Ni-C represented the active state of the enzyme, in which the Ni site was accessible to H^+. The structure of Ni-C center is a Ni(III) with square pyramidal coordination involving four cysteinyl S ligands and one equatorially bound H^-. But in the *A. vinelandii* hydrogenase, the structure of Ni center could be different from that in the *D. gigas* hydrogenase. *A. vinelandii* hydrogenase exhibited weak EPR signals associated with the Ni, unlike the *D. gigas* hydrogenase where strong EPR signals associated with Ni appeared. The catalytic properties of the *A. vinelandii* hydrogenase are also different from those of *D. gigas* hydrogenase. The *A. vinelandii* hydrogenase catalyzes H_2 oxidation efficiently (low K_m & high V_{max}) and only slowly evolves H_2 (Hyman and Arp, 1991). Unfortunately, there is no further paramagnetic spectra data to provide insight to the structure of the Ni center of *A. vinelandii* hydrogenase. But the inhibitors CO, C_2H_2, CN^-, and Cu(II) were shown kinetically to influence the H_2 binding site. To obtain spectral features of the H_2 activation site, the spectral changes in the UV-vis spectra associated with the binding of inhibitors to the *A. vinelandii* hydrogenase were observed in Chapter III and IV of this dissertation. The absorption peaks specific to inhibition of enzyme activity were assumed to arise from the binding of each inhibitor to the H_2 activation site. Binding of C_2H_2 gave rise to the Δ absorption peak at 492 nm, while the binding of CN^- exhibited a Δ trough at 380 nm.

In the past, C_2H_2 was identified as a tightly, slow-binding inhibitor for *A. vinelandii* hydrogenase. In the works presented in Chapter II, C_2H_2 inhibition was recognized to be reversible. The C_2H_2 was recovered upon activation of enzyme activity by incubation with H_2. Upon recovery process, C_2H_2 release proceeded more rapidly than recovery of activity, but the Δ peak at 492 nm disappeared in the same rate as that of

activity restoration (Chapter III). This indicated that the C_2H_2 inhibition of enzyme did not require the continued binding of C_2H_2 to the hydrogenase and would suggest that the C_2H_2 bound to a component of hydrogenase (e.g. Ni or [Fe-S] cluster) and then induced a ligand rearrangement. This ligand arrangement could be very similar to that in the aerobically purified *A. vinelandii* hydrogenase, because the first change in optical feature is the decrease of absorption at 490 nm upon activation of enzyme activity by incubation with H_2.

Previous work with the *D. gigas* hydrogenase indicated that Cu(II) was an inhibitor of hydrogenase (Fernandez, et al., 1989). Chapter IV of this dissertation examined the effects of Cu(II) on the *A. vinelandii* hydrogenase. The result revealed that the Cu(II) when added to the reaction mixture in μM concentration resulted in a very potent inhibition of H_2 oxidation. The other reactions of hydrogenase (production of H_2 and isotope exchange) were also inhibited at similar concentration of Cu(II). The detailed studies in Chapter IV identified the Cu(II) as an inhibitor relative to the H_2 activation site of the hydrogenase and Cu(II) inactivation required the hydrogenase to have a fully functional H_2 activation site. Upon Cu(II) inactivation the [Fe-S] clusters of hydrogenase were destroyed, and a Cu(II)-thiolate complex corresponding to the absorption at 300 nm and 320 nm was formed.

In conclusion, the works presented in this dissertation proved that the H_2 activation site is located in the large subunit of *A. vinelandii* hydrogenase and that reduction of the [Fe-S] clusters depended on the other functional centers (e. g. Ni). In addition, Cu(II) has been characterized as an H_2-activation-site-related inhibitor of *A. vinelandii* hydrogenase. Furthermore, the effects of inhibitors C_2H_2, O_2, CN^-, NO, and Cu(II) on the UV-vis spectra of *A. vinelandii* hydrogenase were observed. These observations, coupled with inhibitory kinetic studies, provided additional insights to the structure and function of hydrogenase.

References

Fernandez, V. M., Rua, M. L., Reyes, P., Cammack, R. & Hatchikian, E. C. (1989) *Eur. J. Biochem. 185*, 449-454.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1987) *Biochemistry 26*, 6447-6454.

Hyndman, L. A., Burris, R. H. & Wilson, P. W. (1953) *J. Bacteriol. 65*, 522-531.

Kow, Y. W. & Burris, R. H. (1984) *J. Bacteriol. 159*, 564-569.

Menon, A. L., Stults, L. W., Robson, R. L. & Mortenson, L. E. (1990) *Gene 96*, 67-74.

Phelps, A. S. & Wilson, P. M. (1941) *Proc. Soc. Exp. Biol. Med. 47*, 473-476.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1992) *FEMS Microbiology Reviews 88*, 109-135.

Sayavedra-Soto, L. A. & Arp, D. J. (1993) *J. Bacteriol. 175*, 3414-3421.

Seefeldt, L. C. (1989) Ph. D thesis, University of California, Riverside.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Teixeira, M., Moura, I., Fauque, G., DerVartanian, D. V., LeGall, J., Peck Jr., H. D.,

Moura, J. J. G. & Huynh, B. H. (1990) *Eur. J. Biochem. 189*, 381-386.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., LeGall, J., Der Vartanian, D. V., Peck, H. D., Jr. & Huynh, B. H. (1989) *J. Biol. Chem. 264*, 16435-16450.

BIBLIOGRAPHY

Adams, M. W. W. (1990a) *FEMS Microbiology Reviews 75*, 219-238.

Adams, M. W. W. (1990b) *Biochim. Biophys. Acta 1020*, 115-145.

Adams, M. W. W. & Hall, D. O. (1979) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 159-169, Goltze, K. G., Gottingen.

Adams, M. W. W., Jin, S. L. C., Chem, J. S. & Mortenson, L. E. (1986) *Biochim. Biophys. Acta 869*, 37-47.

Adams, M. W. W. & Mortenson, L. E. (1984) *J. Biol. Chem. 259*, 7045-7055.

Adams, M. W. W., Mortenson, L. E. & Chen, J. S. (1981) *Biochim. Biophys. Acta. 594*, 105-176.

Albracht, S. P. J., Albrecht-Ellmer, K. J., Schmedding, D. J. M. & Slater, E. C. (1982a) *Biochim. Biophys. Acta 681*, 330-334.

Albracht, S. P. J., Graf, E. G. & Thauer, R. K. (1982b) *FEBS Lett. 140*, 311-314.

Albracht, S. P. J., Kalkman, M. L. & Slater, E. C. (1983) *Biochim. Biophys. Acta 724*, 309-316.

Albracht, S. P. J., Van der Zwaan, J. W. & Fontijn, R. D. (1984) *Biochim. Biophys. Acta 766*, 245-258.

Arp, D. J. (1985) *Arch. Biochem. Biophys. 237*, 504-512.

Arp, D. J. & Burris, R. H. (1979) *Biochim. Biophys. Acta 570*, 221-230.

Arp, D. J., McCollum, L. C. & Seefeldt, L. C. (1985) *J. Bacteriol. 163*, 15-20.

Ballantine, S. P. & Boxer, D. H. (1986) *Eur. J. Biochem. 156*, 277-284.

Bartha, R. & Ordal, E. J. (1965) *J. Bacteriol. 89*, 1015-1019.

Bell, S. H., Dickson, D. P. E., Rieder, R., Cammack, R., Patil, D. S., Hall, D. O. & Rao, K. K. (1984) *Eur. J. Biochem. 145*, 645-651.

Blakeley, R. L., Dixon, N. E. & Zerner, B. (1983) *Biochim. Biophys. Acta. 744*, 219-229.

Bonam, D. & Ludden, P. W. (1987) *J. Biol. Chem. 262*, 2980-2987.

Boursier, M. A., Hanus, F. J., Papen, H., Becker, M. M., Russell, S. A. & Evans, H. J. (1988) *J. Bacteriol. 170*, 5594-5600.

Bowien, B. & Schlegel, H. G. (1981) *Ann. Rev. Microbiol. 35*, 405452.

Brushi, M. & Guerlesquin, F. (1988) *FEMS Microbiol. Rev. 54*, 155-176.

Buchanan, B. B., Lovenberg, W. & Rabinowitz, J. C. (1963) *Proc. Natl. Acad. Sci. USA 49*, 345-353.

Cammack, R., Bagyinka, C. & Kovacs, K. L. (1989) *Eur. J. Biochem. 182*, 357-362.

Cammack, R., Fernandez, V. M. & Schneider, K. (1988) in *Bioinorganic Chemistry of Nickel* (J. R. Lancaster, Ed.) pp 167-190, VCH Publishers, New York.

Cammack, R., Fernandez, V. M. & Schneider, K. (1986) *Biochimie 68*, 85-91.

Cammack, R., Kovacs, K. L., McCracken, J. & Peisach, J. (1989) *Eur. J. Biochem. 182*, 363-366.

Cammack, R., Patil, D., Aguirre, R. & Hatchikian, E. C. (1982) *FEBS Lett. 142*, 289-292.

Cammack, R., Patil, D. S., Hatchikian, E. C. & Fernandez, V. M. (1987) *Biochim. Biophys. Acta 912*, 98-109.

Carney, M. J., Papaefthymiou, G. C., Spartalian, K., Frankel, R. B. & Holm, R. H. (1988a) *J. Am. Chem. Soc. 110*,

Carney, M. J., Papaefthymiou, G. C., Whitener, M. A., Spartalian, K., Frankel, R. B. & Holm, R. H. (1988b) *Inorg. Chem. 27*, 346-352.

Chadwick, B. M. & Sharpe, A. G. (1986) in *Advances in Inorganic Chemistry and Radiochemistry* (H. J. Emeleus and A. G. Sharpe, Ed.) pp 83-176, Academic Press Inc., New York.

Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. & Harrison, P. R. (1986) *EMBO J. 5*, 1221-1227.

Chapman, A., Cammack, R., Hatchikian, C. E., McCracken, J. & Peisach, J. (1988) *FEBS Lett. 242*, 134-138.

Chen, J. C. & Mortenson, L. E. (1992a) *Biochimica et Biophysica Acta 1131*, 122-124.

Chen, J. C. & Mortenson, L. E. (1992b) *Biochimica et Biophysica Acta 1131*, 199-202.

Chen, J. S. & Mortenson, L. E. (1974) *Biochim. Biophys. Acta. 371*, 283-298.

Colbeau, A. & Vignais, P. M. (1983) *Biochim. Biophys. Acta 748*, 128-138.

Conover, R. C., Park, J.-B., Adams, M. W. W. & Johnson, M. K. (1991) *J. Am. Chem. Soc. 113*, 2799-2800.

Cotton, F. A. & Wilkinson, G. (1976) *New York* 412-413.

Coughlan, M. P., Rajagopalan, K. V. & Handler, P. (1969) *J. Biol. Chem. 244*, 2658-2663.

Coyle, C. L. & Stiefel, E. I. (1988) in *The bioinorganic chemistry of nickel* (J. Jack R. Lancaster, Ed.) pp 1-28, VCH, Weinheim.

Cypionka, H. & Dilling, W. (1986) *FEMS Microbiol. Lett. 36*, 257-260.

Czchowski, M. H., He, S. H., Nacro, M., Der Vartanian, D. V., Peck, H. D. J. & LeGall, J. (1984) *Biochem. Biophys. Res. Commun. 125*, 1025-1035.

de Saussure, T. (1839) *Mem. Soc. Phys. Hist. Nat. Geneve. 8*, 163.

Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B. (1975) *J. Am. Chem. Soc. 97*, 4131-4133.

Eidsness, M. K., Scott, R. A., Prickril, B., DerVartanian, D. V., LeGall, J., Moura, I., Moura, J. J. G. & Perk, H. D. J. (1989) *Proc. Natl. Acad. Sci. USA 86*, 147-151.

Eisbrenner, G. & Evans, H. J. (1983) *Annu. Rev. Plant Physiol. 34*, 105-136.

Ensign, S. A., Bonam, D. & PW., L. (1989) *Biochemistry 28*, 4968-4973.

Evans, H. J., Russell, S. A., Hanus, F. J., Papen, H., Sayavedra Soto, L., Zuber, M. & Boursier, P. (1988) in *Nitrogen Fixation: Hundred Years After* (H. Bothe, F. J. de Bruijn and W. E. Newton, Ed.) pp Gustav Fischer, Stuttgart. New York.

Fan, C. L., Gorst, C. M., Ragsdale, S. W. & Hoffman, B. M. (1991) *Biochemistry 30*, 431-435.

Farkas, A., Farkas, L. & Yudkin, J. (1934) *Proc. R. Soc. 115*, 373-379.

Fauque, G., Peck, H. D. J., Moura, J. J. G., Huynh, B. H., Berlier, Y., DerVartanian, D. V., Teixeira, M., Przybyla, A. E., Lespinat, P. A., Moura, I. & LeGall, J. (1988) *FEMS Microbiology Reviews 54*, 299-344.

Fauque, G., Teixeira, M., Moura, I., Lespinat, P. A., Xavier, A. V., Der Vartanian, D. V., Peck, H. D. J., LeGall, J. & Moura, J. J. G. (1984) *Eur. J. Biochem. 142*, 21-28.

Fawcett, T. G., Bernarducci, E. E., Krogh-Jespersen, K. & Schugar, H. J. (1980) *J. Am. Chem. Soc. 102*, 2598-2604.

Fernandez, V. M., Hatchikian, E. C., Patil, D. S. & Cammack, R. (1986) *Biochim. Biophys. Acta 883*, 145-154.

Fernandez, V. M., Rua, M. L., Reyes, P., Cammack, R. & Hatchikian, E. C. (1989) *Eur. J. Biochem. 185*, 449-454.

Fox, J. A., Livingston, D. J., Orme-Johnson, W. H. & Walsh, C. T. (1987) *Biochemsitry 26*, 4219-4227.

Francis, K., Patel, P., Wendt, J. G. & Shanmugan, K. T. (1990) *J. Bacteriol. 172*, 5750-5757.

Friedrich, C. G., Schneider, K. & Friedrich, B. (1982) *J. Bacteriol. 152*, 42-48.

Gitlitz, P. H. & Krasna, A. I. (1975) *Biochemistry 14*, 2561-2568.

Gogotov, I. N. (1986) *Biochimie 68*, 181-187.

Graf, E. G. & Thauer, R. K. (1981) *FEBS Lett. 115*, 285-288.

Harker, A. R., Xu, L., Hanus, F. J. & Evans, H. J. (1984) *J. Bacteriol. 159*, 850-856.

Haschke, R. H. & Campbell, L. L. (1971) *J. Bacteriol. 105*, 249-258.

Hatchikian, E. C., Bruschi, M. & J., L. (1978a) *Biochem. Biophys. Res. Commun. 82*, 451-461.

Hatchikian, E. C., Bruschi, M. & LeGall, J. (1978b) *Biochem. Biophys. Res. Commun. 914*, 299-303.

Hausinger, R. P. (1987) *Microbiological Reviews 51*, 22-42.

He, S. H., Teixeira, M., LeGall, J., Patil, D. S., Der Vartanian, D. V., Huynh, B. H. & Perk, H. D. J. (1989a) *J. Biol. Chem. 264*, 2678-2682.

He, S. H., Woo, S. B., Der Vartanian, D. W., LeGall, J. & Peck, H. D., Jr. (1989b) *Biochem. Biophys. Res. Commun. 161*, 127-133.

Hoberman, H. D. & Rittenberg, D. (1943) *J. Biol. Chem. 147*, 211-227.

Hornhardt, S., Schneider, K. & Schlegel, H. G. (1986) *Biochimie 68*, 15-24.

Houchins, J. P. (1984) *Biochim. Biophys. Acta 768*, 227-255.

Huang, Y.-H., Park, J.-B., Adams, M. W. W. & Johnson, M. K. (1993) *Inorg. Chem. 32*, 375-376.

Huynh, B. H., Patil, D. S., Moura, I., Teixeira, M., Moura, J. J. G., DerVartanian, D. V., Czechowski, M. H., Prickrill, B. C., Peck, H. D. J. & LeGall, J. (1987) *J. Biol. Chem. 262*, 795-800.

Hyman, M. R. & Arp, D. J. (1991) *Biochim. Biophys. Acta 1076*, 165-172.

Hyman, M. R. & Arp, D. J. (1988) *Anal. Biochem. 173*, 207-220.

Hyman, M. R. & Arp, D. J. (1987a) *Biochemistry 26*, 6447-6454.

Hyman, M. R. & Arp, D. J. (1987b) *Appl. Environ. Microbiol. 53*, 298-303.

Hyndman, L. A., Burris, R. H. & Wilson, P. W. (1953) *J. Bacteriol. 65*, 522-531.

Jensen, G. M., Seefeldt, L. C., Arp, D. J. & Stephens, P. J. (1992) *Biochemistry Submitted*,

Jin, S. L. C., Blanchard, D. K. & Chem, J. S. (1983) *Biochim. Biophys. Acta 748*, 8-20.

Joklik, W. K. (1950) *Aust. J. Exp. Biol. Med. Sci. 28*, 331-338.

Juszczak, A., Aono, S. & Adams, M. W. W. (1991) *Journal of Biological Chemistry 266*, 13834-13841.

Kaserer, H. (1906) *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 2*, 681.

Khan, M. F. & Sorenson, J. R. (1991) *Journal of Inorganic Biochemistry 41*, 221-234.

Koch, H. G., Kern, M. & Klemme, J. H. (1992) *FEMS Microbiology Letters 91*, 193-197.

Kojima, N., Fox, J. A., Hausinger, R. P., Daniels, L., Orme-Johnson, W. H. & Walsh, C. T. (1983) *Proc. Natl. Acad. Sci. USA 80*, 378-382.

Kovacs, K. L., Seefeldt, L. C., Tigyi, G., Doyle, C. M., Mortenson, L. E. & Arp, D. J. (1989) *J. Bacteriol. 171*, 430-435.

Kow, Y. W. & Burris, R. H. (1984) *J. Bacteriol. 159*, 564-569.

Krasna, A. I. & Rittenberg, D. (1954) *J. Am. Chem. Soc. 76*, 3015-3020.

Kruger, H. J., Huynh, B. H., Ljungdahl, P. O., Xavier, A. V., Der Vartanian, D. V., Moura, I., Peck, H. D. J., Teixeira, M., Moura, J. J. G. & LeGall, J. (1982) *J. Biol. Chem. 257*, 14620-14623.

Kumar, M., Day, R. O., Colpas, G. J. & Maroney, M. J. (1989) *J. Am. Chem. Soc. 111*, 5974-5976.

Lalla-Maharajh, W. V., Hall, D. O., Cammack, R., Rao, K. K. & LeGall, J. (1983) *Biochem. J. 209*, 445-454.

Lancaster, J. R. J. (1982) *Science 216*, 1324-1325.

Lancaster, J. R. J. (1980) *FEBS Lett. 115*, 285-288.

Leclerc, M., Colbeau, A., Cauvin, B. & Vignais, M. (1988) *Mol. Gen. Genetics 214*, 97-107.

LeGall, J., Dervartanian, D. V., Spilker, E., Lee, J. P. & Perk, H. D., Jr. (1971) *Biochim. Biophys. Acta. 234*, 525-530.

LeGall, J., Ljungdahl, P. O., Moura, I., Peck, H. D. J., Xavier, A., Moura, J. J. G., Teixeira, M., Huynh, B. H. & Der Vartanian, D. V. (1982) *Biochem. Biophys. Res. Commun. 106*, 610-615.

Lespinat, P. A., Berlier, Y., Fauque, G., Czechowski, M. H., Dimon, B. & LeGall, J. (1986) *Biochimie 68*, 55-61.

Li, C., Peck Jr., H. D., LeGall, J. & Przybyla, A. E. (1987) *DNA 6*, 539-551.

Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. H. & Munck, E. (1985) *J. Biol. Chem. 260*, 11160-11173.

Lindahl, P. A., Gorelick, N. J., Munck, E. & Orem-Johnson, W. H. (1987) *J. Biol. Chem. 262*, 14945-14953.

Lindahl, P. A., Kojima, N., Hausinger, R. P., Fox, J. A., Jeo, B. K., Walsh, C. T. & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 3062-3064.

Lissolo, T., Choi, E. S., LeGall, J. & Peck, H. D. J. (1986) *Biochem. Biophys. Res. Commun. 139*, 701-708.

Livingston, D. J., Fox, J. A., Orme-Johnson, W. H. & Walsh, C. T. (1987) *Biochemistry 26*, 4228-4237.

Lovenberg, W., Buchanan, B. B. & Rabinowitz, J. C. (1963) *J. Biol. Chem. 238*, 3899-3913.

Maier, T., Jacobi, A., Sauter, M. & Bock, A. (1993) *Journal of Bacteriology 175*, 630-635.

Maroney, M. J., Copas, G. J. & Bagyinka, C. (1990) *J. Am. Chem. Soc. 112*, 7007-7068.

Mayhew, S. G., Van Dijk, C. & Van der Westen, H. M. (1978) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 125-140, Erich Goltze, Gottingen, FRG.

Menon, A. L., Stults, L. W., Robson, R. L. & Mortenson, L. E. (1990) *Gene 96*, 67-74.

Menon, N. K., Peck Jr., H. D., LeGall, J. & Przybyla, A. E. (1988) *J. Bacteriol. 170*, 4429.

Menon, N. K., Peck Jr., H. D., LeGall, J. & Przybyla, A. E. (1987) *J. Bacteriol. 169*, 5401-5407.

Menon, N. K., Robbins, J., Peck Jr., H. D., Chatelus, C. Y., Choi, E.-S. & Przybyla, A. E. (1990) *J. Bacteriol. 172*, 1969-1977.

Menon, N. K., Robbins, J., Wendt, J. C., Shanmugam, K. T. & Przybyla, A. E. (1991) *Journal of Bacteriology 173*, 4851-4861.

Mortenson, L. E., Valentine, R. C. & Carnahan, J. E. (1962) *Biochem. Biophys. Res. Commun. 7*, 448-452.

Multani, J. S. & Mortenson, L. E. (1972) *Biochim. Biophys. Acta. 256*, 66-70.

Muth, E., Morschel, E. & Klein, A. (1987) *Eur. J. Biochem. 109*, 571-577.

Nag, K. & Chakravorty, A. (1980) *Coordination Chemistry Reviews 33*, 87-147.

Nakos, G. & Mortenson, L. E. (1971a) *Biochim. Biophys. Acta 227*, 576-583.

Nakos, G. & Mortenson, L. E. (1971b) *Biochemistry 10*, 2442-2449.

Niviere, V., Forget, N., Gayda, J. P. & Hatchikian, E. C. (1986) *Biochem. Biophys. Res. Commun. 139*, 658-665.

Peck, H. D. J. & Gest, H. (1957) *J. Bacteriol. 73*, 569-580.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.

Phelps, A. S. & Wilson, P. M. (1941) *Proc. Soc. Exp. Biol. Med. 47*, 473-476.

Pinkwart, M., Schneider, K. & Schlegel, H. G. (1983) *Biochim. Biophys. Acta. 745*, 267-278.

Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. (1992) *FEMS Microbiology Reviews 88*, 109-135.

Reeve, J. N. & Beckler, G. S. (1990) *FEMS Microbiol. Revs. 87*, 425-430.

Rieder, R., Cammack, R. & Hall, D. O. (1984) *Eur. J. Biochem. 145*, 637-643.

Ruhland, W. (1922) *Ber. Botan. Ges. 40*, 180-184.

Sadana, J. C. & Jagannathan, V. (1956) *Biochim. Biophys. Acta 19*, 446-452.

Sadana, J. C. & Rittenberg, D. (1963) *Proc. Natl. Acad. Sci. USA 50*, 900-904.

Sawers, R. G. & Boxer, D. H. (1986) *Eur. J. Biochem. 156*, 265-275.

Sayavedra-Soto, L. A. & Arp, D. J. (1993) *J. Bacteriol. 175*, 3414-3421.

Sayavedrasoto, L. A. & Arp, D. J. (1992) *Journal of Bacteriology 174*, 5295-5301.

Sayavedra-Soto, L. A., Powell, G. A., Evans, H. J. & Morris, R. O. (1988) *Proc. Natl. Acad. Sci. USA 85*, 8395-8399.

Schink, B. & Schlegel, H. G. (1979) *Biochim. Biophys. Acta. 567*, 315-324.

Schlegel, H. G. (1954) *Flora 141*, 1-15.

Schneider, K. & Cammack, R. (1978) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (H. G. Schlegel and K. Schneider, Ed.) pp 221-234, Erich Goltze, Gottingen, FRG.

Schneider, K., Cammack, R. & Schlegel, H. G. (1984) *Eur. J. Biochem. 142*, 75-84.

Schneider, K. & Piechulla, B. (1986) *Biochimie 68*, 5-13.

Schneider, K., Patil, D. S. & Cammack, R. (1983) *Biochim. Biophys. Acta 748*, 353-361.

Schoenmaker, G. S., Oltmann, L. F. & Stouthamer, A. H. (1979) *Biochim. Biophys. Acta. 567*, 511-521.

Scott, R. A., Wallin, S. A., Czechowski, M., Der Vartanian, D. V., Le Gall, J., Peck, H. D. J. & Moura, I. (1984) *J. Am. Chem. Soc. 106*, 6864-6865.

Seefeldt, L. C. (1989) Ph. D thesis, University of California, Riverside.

Seefeldt, L. C. & Arp, D. J. (1989a) *J. Bacteriol. 171*, 3298-3303.

Seefeldt, L. C. & Arp, D. J. (1989b) *Biochemistry 28*, 1588-1596.

Seefeldt, L. C. & Arp, D. J. (1986) *Biochimie 68*, 25-34.

Seefeldt, L. C., Fox, C. A. & Arp, D. J. (1986) *J. Biol. Chem. 261*, 10688-10694.

Seefeldt, L. C., McCollum, L. C., Doyle, C. M. & Arp, D. J. (1987) *Biochim. Biophys. Acta 914*, 299-303.

Serebryakova, L. T., Zorin, N. A. & Gogotov, I. N. (1987) *Biochemistry Engl. Transl. Biokhimiya 52*, 779-785.

Shug, A. L., Wilson, P. W., Green, D. E. & Mahler, H. R. (1954) *J. Am. Chem. Soc. 76*, 3355-3356.

Sigel, H. & Martin, R. B. (1982) *Chem. Rev. 82*, 385-426.

Solomonson, L. P. (1981) in *Cyanide in Biology* (B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing, Ed.) pp 11-28, Academic Press, Inc., New York.

Stephenson, M. & Stickland, L. H. (1931) *Biochem. J. 25*, 205-215.

Stults, L. W., O'Hara, E. B. & Maier, R. J. (1984) *J. Bacteriol. 159*, 153-158.

Sun, J. H. & Arp, D. J. (1991) *Arch. Biochem. Biophys. 287*, 225-233.

Sun, J. H., Hyman, M. R. & Arp, D. J. (1992) *Biochemistry 31*, 3158-3165.

Sweeney, W. V. (1980) *Ann. Rev. Biochem. 49*, 139-161.

Tamiya, N., Kondo, Y., Kameyama, T. & Akabori, E. (1955) *J. Biochem. 42*, 613-614.

Tan, S. L., Fox, J. A., Kojima, N., Walsh, C. T. & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 3064-3066.

Teixeira, M., Fanque, G., Moura, I., Lespinat, P. A., Berlier, Y., Pickrill, B., Peck, H. D. J., Xavier, A. V., LeGall, J. & Moura, J. J. G. (1987) *Eur. J. Biochem. 167*, 47-58.

Teixeira, M., Moura, I., Fauque, G., Czechowski, M., Berlier, Y., Lespinat, P. A., LeGall, J., Xavier, A. V. & Moura, J. J. G. (1986) *Biochimie. 68*, 75-84.

Teixeira, M., Moura, I., Fauque, G., DerVartanian, D. V., LeGall, J., Peck Jr., H. D., Moura, J. J. G. & Huynh, B. H. (1990) *Eur. J. Biochem. 189*, 381-386.

Teixeira, M., Moura, I., Xavier, A. V., Huynh, B. H., Der Vartanian, D. V., Peck, H. D., Jr., LeGall, J. & Moura, J. J. G. (1985a) *J. Biol. Chem. 260*, 8942-8950.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., Fauque, G., Pickril, B. & LeGall, J. (1985b) *Rev. Port. Quim. 27*, 194-195.

Teixeira, M., Moura, I., Xavier, A. V., Moura, J. J. G., LeGall, J., Der Vartanian, D. V., Peck, H. D., Jr. & Huynh, B. H. (1989) *J. Biol. Chem. 264*, 16435-16450.

Tieghem, E., Van Dael, H. & Van Cauwelaert, F. (1991) *Journal of Inorganic Biochemistry 42*, 119-131.

Tigyi, G., Bagyinka, C. & Kovacs, K. C. (1986) *Biochimie 68*, 69-74.

Torres, V., Ballesteros, A. & Fernandez, V. M. (1986) *Arch Biochem. Biophys. 245*, 174-178.

Tran-Betcke, A., Warnecke, U., Boecker, C., Zaborosch, C. & Friedrich, B. (1990) *J. Bacteriol. 172*, 2920-2929.

Unden, G., Bocher, R., Knecht, J. & Kroger, A. (1982) *FEBS Lett. 145*, 230-234.

van der Werf, A. N. & Yates, M. G. (1978) in *Hydrogenases: Their catalytic activity, structure and function* (H. G. Schlegel and K. Schneider, Ed.) pp 307-326, Erich Goltze KG.

Van der Westen, H. M., Mayhew, S. G. & Veeger, C. (1978) *FEBS Lett. 86*, 122-126.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Mul, P. (1987) *Eur. J. Biochem. 169*, 377-384.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Roelofs (1986) *Biochim. Biophys. Acta 872*, 208-215.

Van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D. & Slater, E. C. (1985) *FEBS Lett. 179*, 271-277.

Van der Zwaan, J. W., Coremans, J. M. C. C., Bouwens, E. C. M. & Albracht, S. P. J. (1990) *Biochim. Biophys. Acta. 1041*, 101-110.

Van Heerikhuizen, H., Albracht, S. P. J., Slater, E. C. & Van Rheenen, P. S. (1981) *Biochim. Biophys. Acta 657*, 26-39.

Voordouw, G. & Brenner, S. (1985) *Eur. J. Biochem. 148*, 515-520.

Wallace, E. F. & Rabirowitz, J. C. (1971) *Arch. Biochem. Biophys. 146*, 400-409.

Walsh, C. T. & Orme-Johnson, W. H. (1987) *Biochemistry 26*, 4901-4906.

Waring, W. S. & Werkman, C. H. (1944) *Arch. Biochem. 4*, 75-87.

Wendel, A., Pilz, W., Ladenstein, R., Sawatzki, G. & Weser, U. (1975) *Biochim. Biophys. Acta 377*, 211-215.

Yagi, T., Kimura, K., Daidoji, H., Sakai, F., Tamura, S. & Inokuchi, H. (1973) *J. Biochem. 79*, 661-677.

Yamazaki, S. (1982) *J. Biol. Chem. 257*, 7926-7929.

Yates, M. G., Ford, C. M., Tibelius, K. H., Campbell, F., Arp, D. J. & Seefeldt, L. C. (1988) in *Nitrogen Fixation: Hundred Years After* (H. Bothe, F. J. de Bruijn and W. E. Newton, Ed.) pp 263-269, Gustav Fischer, New York.

Zinoni, F., Birkmann, A., Stadtman, T. C. & Bock, A. (1986) *Proc. Natl. Acad. Sci. USA 83*, 4650-4654.

Zirngibl, C., Van Dongen, W., Schwoerer, B., Von Buenau, R., Richter, M., Klein, A. & Thauer, R. K. (1992a) *Eur. J. Biochem. 208*, 511-520.

Zorin, N. A. (1986) *Biochimie 68*, 97-101.

APPENDIX I

FURTHER CHARACTERIZATION OF ACETYLENE INHIBITED HYDROGENASE

In this work, the ^{14}C-acetylene labeled hydrogenase was cleaved by using trypsin. The fragments with radioactivity was isolated by using HPLC equipped with a Vydac C_{18} reverse-phase column, and was further purified by reloading fractions to the same column. However, we could not get any peptides from the pooled radioactive fractions. This suggested that the acetylene bound fragment could be insoluble. Also, we isolated the radio-labeled fragments by using limited proteolysis, SDS-PAGE, and electrophoresis blot to a PVDF membrane. One radioactive bands cut from the PVDF membrane was sequenced at N-terminus. It showed that at least 5 fragments constituted this band. Limited to the release of bound acetylene from the protein, we could not detect the radioactivity in the fractions recovered from sequencing. The hydrogenase is easily oxidized under nitrogen gas. This auto-oxidized form of hydrogenase is not sensitive to acetylene inhibition. Addition of DTT, EDTA or dihydrogen releases the auto-oxidization, and consequently, it makes the enzyme be sensitive to the acetylene inhibition. This indicated that the acetylene inhibition depended on the oxidation state of the enzyme. In addition, the acetylene inhibition is pH dependent. At a lower pH (i.e., pH4), the rate of acetylene inhibition is faster than at higher pHs. When the pH is over 8, little inhibition is observed over 30 min incubation. Consistent with these observations, the higher pH stimulates the rate of hydrogenase recovery from acetylene inhibition.