Acetylene Inhibition of *Azotobacter vinelandii* Hydrogenase: Acetylene Binds Tightly to the Large Subunit†

Jin-Hua Sun, Michael R. Hyman, and Daniel J. Arp*

*Laboratory for Nitrogen Fixation Research, Oregon State University, 2082 Cordley Hall, Corvallis, Oregon 97331-2902

Received October 18, 1991; Revised Manuscript Received January 6, 1992

**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 C₂H₂ proceeds more rapidly than the recovery of activity, which indicates that release of C₂H₂ 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 C₂H₂. Acetylene, or a derivative, remains bound to the large subunit of this dimeric hydrogenase. Radioactivity is lost from ¹⁴C₂H₂-inhibited protein during recovery. The inhibition is remarkably specific for C₂H₂; propyne, butyne, and ethylene are not inhibitors.

The nitrogen-fixing bacterium *Azotobacter vinelandii* expresses a single, membrane-bound hydrogenase. The physiological function of this enzyme is to oxidize the H₂ produced by nitrogenase during the reduction of N₂ to NH₃. *A. vinelandii* hydrogenase efficiently scavenges the H₂ produced in situ by nitrogenase. Hydrogenase is a dimer composed of two identical subunits (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 H₂ 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. ⁷⁷Se 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

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† This work was supported by U.S. Department of Energy Grant No. DEFGG-90ER20013 and the Oregon Agricultural Experiment Station.

*To whom correspondence should be addressed.

**Abbreviations:** EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.
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 (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 H₂ oxidation by hydrogenase and of probing the role of the metal centers in catalysis. A number of inhibitors of *A. vinelandii* hydrogenase have been characterized, including O₂ (Seefeldt & Arp, 1989b), CN⁻ (Seefeldt & Arp, 1989a), and NO (Hyman & Arp, 1991). This paper deals with the inhibitor C₂H₂. Smith et al. (1976) first recognized the ability of C₂H₂ to inhibit hydrogenase in intact *Azotobacter chroococum* cells. Yates and co-workers (van der Werf & Yates, 1978) demonstrated that the inhibition required preincubation of hydrogenase in the absence of H₂ and that the inhibition was reversible. Hyman and Arp (1987a) provided a thorough characterization of the kinetic mechanism of C₂H₂ inhibition. Acetylene is a slow-binding, active-site-directed inhibitor of *A. vinelandii* hydrogenase. H₂ is a potent and competitive protectant against inhibition by C₂H₂. He et al. (1989a) showed that the NiFe hydrogenase of *D. gigas* and the NiFeSe hydrogenase of *D. baculatus* are inhibited by C₂H₂, while the “Fe-only” hydrogenase of *Desulfotibrio vulgaris* is not inhibited by C₂H₂. This supported the idea that C₂H₂ reacts with Ni in NiFe hydrogenases (He et al., 1989a; Hyman & Arp, 1987a). However, 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 C₂H₂.

Despite the interest in C₂H₂ as an inhibitor of hydrogenases, several fundamental questions regarding the mechanism of C₂H₂ inhibition remain. For example, it has not been demonstrated that C₂H₂ remains bound to hydrogenase following inhibition nor has it been demonstrated that C₂H₂, rather than a derivative of C₂H₂, is released during recovery from C₂H₂ inhibition. We have proposed that C₂H₂ might act as an analogue of H₂ (Hyman & Arp, 1987a). This raises the possibility that C₂H₂, like H₂, is activated by hydrogenase and transformed to another compound. Perhaps the transformed compound is the actual inhibitor. Alternatively, the transformed C₂H₂ might be released from the enzyme, leaving behind an inactive hydrogenase, or the transformed C₂H₂ could remain bound while hydrogenase is inhibited and then be released as C₂H₂ during recovery. In this work, we have further investigated the mechanism of C₂H₂ inhibition of *A. vinelandii* hydrogenase. The inhibition was specific for C₂H₂, and no transformation of C₂H₂ 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 C₂H₂ and most likely H₂ as well bind to the large subunit of this Ni-containing hydrogenase.

**MATERIALS AND METHODS**

**Materials.** Residual O₂ was removed from H₂ and N₂ (>99.9% purity) by passage over a heated copper-based catalyst (R-3-11, Chemical Dynamics Corp., South Plainfield, NJ). Gas from an acetylene cylinder (99.6%) was vented until no H₂ gas was detectable by gas chromatography. Acetylene was further purified cryogenically as described (Hyman & 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 purchased 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 & Arp, 1989b). The hydrogenase was purified from membranes as previously described (Sun & Arp, 1991). All steps were performed under anaerobic conditions and in the presence of 2 mM Na₂S₂O₄.

**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 and 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 of protein)⁻¹ (pH 6.0, methylene blue assay at 30 °C).

**SDS–PAGE.** Discontinuous vertical slab gels (10 or 12% (w/v) acrylamide; 10 × 6.0 × 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 (12300). Proteins were visualized by staining with Coomassie blue.

**Incubation Procedures for C₂H₂ Inhibition.** Incubations of hydrogenase with C₂H₂ were carried out in shorted 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 C₂H₂ (101 kPa) or a mixture of C₂H₂ and N₂. 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 Na₂S₂O₄ in 50 mM Tris-HCl (pH 7.5). Each vial also contained an O₂ scavenger (0.5 mL of 0.1 M Na₂S₂O₄ 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 C₂H₂ Inhibition.** To allow hydrogenase to recover from inhibition by C₂H₂, unbound C₂H₂ 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 H₂. The outer section of the vial contained an O₂ scavenger (see above). The Na₂S₂O₄ concentration in the enzyme sample was raised to 4 mM by addition of Na₂S₂O₄ 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 H₂ oxidation was determined as a measure of hydrogenase activity (Arp & Burris, 1981).

**Fluorography of ¹⁴C-Labeled Polypeptides.** For fluorography of ¹⁴C-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 & 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 semidry blotter. An enzyme-linked immunosorbent assay was performed on the nitrocellulose sheet as described (Birckett et al., 1985) with antisera (200-fold dilution) prepared against *B. japonicum* hydrogenase large subunit or small subunit. Peroxidase-conjugated goat antirabbit antibodies were used diluted 2000-fold (Seefeldt & Arp, 1987).

**¹⁴C₂H₂ Preparation.** ¹⁴C₂H₂ was synthesized from Ba¹⁴CO₃ by a modification of a previously described method (Hyman & Arp, 1990). Briefly, 2.5 mCi of Ba¹⁴CO₃ (specific activity = 56 mCi/mmol) was thermally fused with approximately 300 mg of finely shredded Ba metal in a Pyrex ignition tube. The fused material containing Ba¹⁴C₂ was transferred to a glass serum vial (160 mL). The vial was stoppered and flushed with Ar for 10 min to deoxygenate the vial. The metal was stoppered and flushed with Ar for 10 min to deoxygenate the vial. The metal was then allowed to warm, and the condensed C₂D₂ was sublimed to fill evacuated serum vials connected to the collection vessel. This method of acetylene generation did not make use of the previously described H₂SO₄ trap (Hyman & Arp, 1987b) so as to eliminate proton exchange between C₂D₂ and the acid. Protonated acetylene (C₂H₂) used for rate comparisons was generated in exactly the same way except that D₂O was replaced with H₂O.

**RESULTS**

**Acetylene Is Released from Hydrogenase during Recovery from Inhibition.** Previous studies demonstrated that inhibition of hydrogenases by C₂H₂ is time-dependent and reversible (van der Werf & Yates, 1978; Hyman & Arp, 1987a). However, these studies did not consider the possibility that C₂H₂ is transformed by hydrogenase to another compound during the inhibition. To test this possibility, the reaction mixtures following inhibition of hydrogenase with C₂H₂ 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 hydrocarbons (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 C₂H₂ was not converted to another compound by hydrogenase.

To confirm that C₂H₂ was not transformed by hydrogenase, a hydrogenase sample was incubated with C₂H₂, the unbound C₂H₂ was removed, and the release of C₂H₂ during recovery of activity was determined. Hydrogenase was inhibited with C₂H₂ (50 kPa, 20 h) until the activity had decreased to less than 1% of the original activity. Unbound C₂H₂ 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 H₂. Activity slowly recovered during the next 70 h to 100% of the original value (Figure 1). During this time, samples of the gas phase were removed and analyzed by gas chromatography. The results (Figure 1) revealed that a gaseous compound that comigrated with C₂H₂ was released during the recovery of activity from C₂H₂ inhibition. To further confirm the identity of this compound as C₂H₂, AgNO₃ (which complexes selectively with N-terminal alkynes) was added to the reaction vials, and this resulted in the disappearance of the compound that coeluted with C₂H₂. A hydrogenase sample incubated in the presence of H₂ and C₂H₂ was not inhibited and maintained full activity throughout the recovery period. Only a small amount of C₂H₂ was released from this sample during the recovery period (Figure 1). For the hydrogenase sample inhibited with C₂H₂, the amount of C₂H₂ released into the gas phase was 1.29 mmol, which compares to the 1.27 mmol of hydrocarbon used in the experiment. It is noteworthy that the kinetics of release of C₂H₂ into the gas phase did not correspond with the recovery of activity, rather C₂H₂ release proceeded more rapidly than recovery of activity. For example,

**C₂H₂ Binding Studies.** Purified *A. vinelandii* hydrogenase (175 μg) was incubated in 60 μL of 20 mM Tris-HCl, 2 mM EDTA, and 2 mM Na₃S₂O₄ (pH 7.5) under a gas phase of 2.8 kPa ¹⁴C₂H₂ (determined from the radioactivity in the aqueous solution equilibrated with the gas phase) and 98 kPa Ar for 24 h, which resulted in 67% inhibition of hydrogenase activity. The majority of the unbound C₂H₂ 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 x 0.6 cm diameter) equilibrated with H₂-purged 20 mM Tris-HCl, 2 mM EDTA, and 2 mM Na₃S₂O₄ (pH 7.5). As the column was developed, fractions of approximately 100 μL were collected in N₂-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 ¹⁴C window. Counting efficiency was determined to be 80%. The remainder of each fraction was injected into an activation vial (see Recovery of Activity following C₂H₂ Inhibition, above) and was incubated with 101 kPa H₂ for 50 h. The ¹⁴C₂H₂-binding experiment was repeated but with the inclusion of H₂ (20 kPa) during the initial incubation. The H₂ prevented C₂H₂ inhibition (Hyman & Arp, 1987a); the sample retained 97% of the initial activity during the incubation in the presence of C₂H₂.

**D₂ Preparation.** Deuterated acetylene (C₂D₂) was generated by adding 10 mL of D₂O (99% purity) to 3 g of CaC₂ in a stopped side-arm flask (50 mL). The resulting gas was collected in a cryogenic gas purification vessel (Hyman & Arp, 1987b) immersed in liquid N₂. After the hydrolysis of the CaC₂ was complete, the collection vessel was evacuated to remove noncondensed contaminating gases. The collection vessel was then allowed to warm, and the condensed C₂D₂ was sublimed to fill evacuated serum vials connected to the collection vessel. This method of acetylene generation did not make use of the previously described H₂SO₄ trap (Hyman & Arp, 1987b) so as to eliminate proton exchange between C₂D₂ and the acid. Protonated acetylene (C₂H₂) used for rate comparisons was generated in exactly the same way except that D₂O was replaced with H₂O.

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**FIGURE 1:** Release of C$_2$H$_2$ from and recovery of activity by C$_2$H$_2$-inhibited hydrogenase. C$_2$H$_2$-inhibited hydrogenase (50 µL, 2.54 mg/mL protein) was passed through a Sephadex G-25 column and eluted with 50 mM Tris-HCl (pH 7.5) under Ar to remove the unbound C$_2$H$_2$. 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$_2$H$_2$ was quantified by gas chromatography (Q). An additional sample (1 µL) was removed for determination of hydrogenase activity (O). The experiment was repeated, except that the hydrogenase was incubated in the presence of C$_2$H$_2$ (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$_2$H$_2$ was quantified by gas chromatography (A).

Most of the C$_2$H$_2$ (89%) had been released within 20 h, 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$_2$H$_2$ present in the vial at time taken as t = 0 (note that this C$_2$H$_2$ must have coeluted with the hydrogenase and that the quantity was substantially greater than in the uninhibited control). Apparently, a substantial amount of C$_2$H$_2$ 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 (Figure 1) indicate that C$_2$H$_2$ (or a derivative) binds tightly to hydrogenase during inhibition. To directly demonstrate the binding of C$_2$H$_2$, or a derivative of C$_2$H$_2$, to hydrogenase, we inhibited hydrogenase with $^{14}$C$_2$H$_2$ 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$_2$H$_2$ and to remove interfering contaminants such as H$_2$. The low association rate constant for binding of C$_2$H$_2$ to hydrogenase indicates an exceptionally sluggish interaction (Schloss, 1988), which demands that high partial pressures of C$_2$H$_2$ (50–101 kPa) be used in order to obtain rapid and complete inhibitions (>90% inhibition in <1 h). However, it is not practical to use high concentrations of purified $^{14}$C$_2$H$_2$ of high specific activity. Therefore, the inhibitions took place in low concentrations of $^{14}$C$_2$H$_2$ (2–5 kPa) for long periods of time (typically 24 h) and did not proceed to completion. Finally, all manipulations required strictly anaerobic conditions.

When hydrogenase was incubated in the presence of $^{14}$C$_2$H$_2$ (2.8 kPa) for 24 h, the activity was inhibited by 67%. Following the removal of the majority of the unbound $^{14}$C$_2$H$_2$ 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$_2$H$_2$ from the protein. Determinations of the radioactivity in the column fractions revealed that $^{14}$C from $^{14}$C$_2$H$_2$ coeluted with hydrogenase activity (Figure 2). When H$_2$ was included during the initial incubation with $^{14}$C$_2$H$_2$, 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 was inhibited by C$_2$H$_2$. The radioactivity in fractions one through four corresponded to 0.58 nmol of $^{14}$C$_2$H$_2$. The substoichiometric amount of C$_2$H$_2$ probably reflects the release of some bound C$_2$H$_2$ from hydrogenase during the time required to process the sample. This is consistent with the experiment described above (Figure 1) where the sample taken at the first time point already contained a significant amount of C$_2$H$_2$. In the experiment described in Figure 2, the C$_2$H$_2$ 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$_2$H$_2$ to hydrogenase, samples of the enzyme that had been inhibited with $^{14}$C$_2$H$_2$ were treated with SDS sample buffer, electrophoresed, and then fluorographed. The fluorogram revealed two bands of radioactivity associated with $^{14}$C$_2$H$_2$-inhibited hydrogenase (Figure 3). The bands were greatly diminished in intensity when the hydrogenase was incubated with H$_2$ and $^{14}$C$_2$H$_2$ prior to electrophoresis. Of the two bands of radioactivity revealed in the fluorogram (Figure 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 (Figure 3, lane 2), and the extent of degradation increased during the long incubation period whether in the presence (lane 3) or absence...
The time dependency of the binding of 14C from 14C2H2 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 14C2H2, a time-dependent increase in the level of radioactivity on the gel was observed (Figure 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 14C from 14C2H2 and loss of activity are, indeed, related.

**14C Is Released from Hydrogenase during Recovery from Inhibition by 14C2H2.** The results of Figure 1 indicated that C2H2 was released from hydrogenase during recovery from C2H2 inhibition. Therefore, we expected that the 14C bound to hydrogenase should also be released during the recovery from inhibition by 14C2H2. To test this expectation, hydrogenase was inhibited with 14C2H2, and then activity was allowed to recover following removal of the unbound 14C2H2.
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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$_2$H$_2$ was released during the recovery period (Figure 5). The time course of recovery (Figure 5A) and the amount of label remaining with the protein (Figure 5B) throughout the recovery period are shown. The label was released from both the large subunit and the weak C$_2$H$_2$-induced band.

This experiment also confirmed an important point indicated by the experiment reported in Figure 1, namely, that the amount of activity recovered and the amount of label lost were not proportional throughout the time course. This was most evident in the first 3 h 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 time-dependent inhibition of hydrogenase activity, similar to the inhibition by C$_2$H$_2$. 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$_2$H$_2$ is an inhibitor, e.g., nitrogenase and ammonia monoxygenase, other alkynes in addition to C$_2$H$_2$ are inhibitors (Hyman & 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$_2$H$_2$ 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$_2$H$_2$ (1.7 kPa) which contaminated the 1-butyne. When C$_2$H$_2$ (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$_2$H$_2$. These results, taken together with the results described above, indicate that the inhibition by C$_2$H$_2$ is remarkably specific for C$_2$H$_2$.

Acetylene as an Analogue of H$_2$. As described below, several lines of evidence support the idea that C$_2$H$_2$ 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 & Burris, 1981). To determine if there is an observable kinetic isotope effect on the rate of acetylene inhibition, both C$_2$H$_2$ and C$_2$D$_2$ 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$_2$O, and C$_2$D$_2$ would be expected to exchange with solvent protons to form C$_2$HD and C$_2$H$_2$. 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$_2$D$_2$ exchanged in 24 h) relative to the rates of inhibition at the pH used in the experiment. When hydrogenase was exposed to either C$_2$D$_2$ or C$_2$H$_2$, 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 & Arp, 1989b). If C$_2$H$_2$ and H$_2$ bind analogously to hydrogenase, then perhaps C$_2$H$_2$ could also protect hydrogenase from irreversible inactivation by O$_2$. To test this possibility, hydrogenase was first inhibited with C$_2$H$_2$ (101 kPa for 4 h, 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 h. This length of exposure to

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**FIGURE 5:** Time course of the loss of $^{14}$C from and recovery of activity by hydrogenase inhibited with $^{14}$C$_2$H$_2$. $^{14}$C$_2$H$_2$-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$_2$ or 101 kPa C$_2$H$_2$. 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$_2$H$_2$ (○). 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 C$_2$H$_2$. (Panel C) Fluorogram for hydrogenase incubated in C$_2$H$_2$.

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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$_2$H$_2$ over the same time period required for recovery of activity (Figure 5). Although the enzyme remained inhibited because of the continued presence of C$_2$H$_2$, the amount of label associated with the protein decreased with time (Figure 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$_2$H$_2*. The possibility was considered that other compounds might also cause a time-dependent inhibition of hydrogenase activity, similar to the inhibition by C$_2$H$_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).

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air was sufficient for complete inactivation of a sample not pretreated with C\textsubscript{2}H\textsubscript{2} (Seefeldt & Arp, 1989b). The air was then evacuated and replaced with H\textsubscript{2} (101 kPa), and the enzyme was incubated for an additional 52 h (the time required for recovery from C\textsubscript{2}H\textsubscript{2} inhibition). During this incubation, hydrogenase activity was recovered (99–103% of the original activity). This result indicates that C\textsubscript{2}H\textsubscript{2}, like H\textsubscript{2}, can protect hydrogenase from irreversible inactivation by O\textsubscript{2}.

**DISCUSSION**

Acetylene inhibits a number of metalloenzymes, including nitrogense, ammonia, and methane monooxygenases, nitrous oxide reductase and hydrogenase (Hyman & Arp, 1988). The mechanism of the inhibition varies with the enzyme. For example, C\textsubscript{2}H\textsubscript{2} is an alternative substrate for nitrogense which inhibits N\textsubscript{2} reduction by competing for reductant and ATP. With ammonia and methane monooxygenases, C\textsubscript{2}H\textsubscript{2} is a mechanism-based inactivator. The catalytic activity of the monooxygenases activates C\textsubscript{2}H\textsubscript{2} to a reactive intermediate which binds irreversibly to the enzyme. For hydrogenases, C\textsubscript{2}H\textsubscript{2} was described as an active-site-directed, slow-binding inhibitor (Hyman & Arp, 1987a). The slow binding of C\textsubscript{2}H\textsubscript{2} to hydrogenase results in a time dependency of the inhibition. The inhibition is reversible, albeit slowly, when the C\textsubscript{2}H\textsubscript{2} is removed. The following observations have led to the idea that C\textsubscript{2}H\textsubscript{2} acts as an analogue of H\textsubscript{2}. (1) H\textsubscript{2} protects hydrogenase from inhibition by C\textsubscript{2}H\textsubscript{2}, and the interaction of H\textsubscript{2} and C\textsubscript{2}H\textsubscript{2} with hydrogenase is competitive (Hyman & Arp, 1987a). (2) Both H\textsubscript{2} activation and C\textsubscript{2}H\textsubscript{2} inhibition require catalytically competent enzyme (Hyman et al., 1988). (3) Neither H\textsubscript{2} or C\textsubscript{2}H\textsubscript{2} alters the EPR spectrum associated with dithionite-reduced hydrogenase, and both H\textsubscript{2} and C\textsubscript{2}H\textsubscript{2} cause a similar change in the EPR spectrum of O\textsubscript{2}-inhibited hydrogenase (Seefeldt, 1989). (4) Both H\textsubscript{2} (Seefeldt & Arp, 1989b) and C\textsubscript{2}H\textsubscript{2} (this work) protect hydrogenase from irreversible inactivation by O\textsubscript{2}. In contrast, CO (another hydrogenase inhibitor which is competitive vs H\textsubscript{2}) does not protect hydrogenase from irreversible inactivation by O\textsubscript{2} (Seefeldt & Arp, 1989b).

Given these similarities, we considered the possibility that C\textsubscript{2}H\textsubscript{2} acts as an analogue of H\textsubscript{2}, but that C\textsubscript{2}H\textsubscript{2} protects hydrogenase. Although this hypothesis is consistent with the reversible nature of the inhibition and binding of C\textsubscript{2}H\textsubscript{2} to native protein, the determination of the relative amount of C\textsubscript{2}H\textsubscript{2} label associated with hydrogenase during the recovery (Figure 5) was surprisingly given the reversible nature of the inhibition and binding of C\textsubscript{2}H\textsubscript{2} to native protein. The determination of the relative amount of C\textsubscript{2}H\textsubscript{2} label associated with hydrogenase during the recovery (Figure 5) was surprising given the reversible nature of the inhibition and binding of C\textsubscript{2}H\textsubscript{2} to native protein. Further, none of the additional denaturating treatments resulted in the release of the label. Apparently, denaturation "locks" the C\textsubscript{2}H\textsubscript{2}-derived label onto the protein, perhaps through a covalent interaction of the C\textsubscript{2}H\textsubscript{2} with hydrogenase. The mechanism of inhibition of hydrogenase by C\textsubscript{2}H\textsubscript{2} may involve the covalent attachment of C\textsubscript{2}H\textsubscript{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.

**Acetylene Binds Reversibly to A. vinelandii Hydrogenase.**

The results of this work (Figures 1, 2, and 3) clearly demonstrate that C\textsubscript{2}H\textsubscript{2} (or a derivative of C\textsubscript{2}H\textsubscript{2}) does, indeed, bind tightly to A. vinelandii hydrogenase. Although precise quantitation is difficult, the analysis of the data from Figures 1 and 2 support a 1:1 stoichiometry of C\textsubscript{2}H\textsubscript{2} bound to hydrogenase. We had previously shown that purified hydrogenase could at least partially recover activity when C\textsubscript{2}H\textsubscript{2} was removed (Hyman & Arp, 1987a). In this work, we demonstrate that the recovery can be complete (e.g., Figures 1 and 5), but requires from 15 to 70 h to recover fully. The reason for the variability of recovery times is not known. During the recovery of activity from C\textsubscript{2}H\textsubscript{2} inhibition, C\textsubscript{2}H\textsubscript{2} was released from the native enzyme (Figure 1). However, the release of C\textsubscript{2}H\textsubscript{2} and the recovery of activity were not coincident (Figure 1 and 5). Acetylene was released more rapidly than activity was recovered. This result was also demonstrated by two independent techniques, namely, measurement by gas chromatography of the C\textsubscript{2}H\textsubscript{2} released during the time course of recovery (Figure 1) and determination of the relative amount of C\textsubscript{2}H\textsubscript{2} label associated with hydrogenase during the recovery (Figure 5). Apparently, C\textsubscript{2}H\textsubscript{2} release from the enzyme is a requirement for, but not in itself sufficient for, recovery of activity. The release of C\textsubscript{2}H\textsubscript{2} prior to recovery of activity would suggest that C\textsubscript{2}H\textsubscript{2} behaves as an analogue of H\textsubscript{2} and that label from C\textsubscript{2}H\textsubscript{2} to the large subunit (Figure 3). This is consistent with our model. While the continued presence of C\textsubscript{2}H\textsubscript{2} prevents recovery of activity, this experiment does not reveal if this occurs by direct binding of C\textsubscript{2}H\textsubscript{2} to E' or follows the reaction sequence E → E' → E → E''.

**14C Label from 14C2H2 Is Bound to the Large Subunit of A. vinelandii Hydrogenase.**

Analysis by SDS–PAGE and fluorography of 14C2H\textsubscript{2}-inhibited hydrogenase revealed that label was associated with the large subunit (Figure 3). This result was surprising given the reversible nature of the inhibition and binding of C\textsubscript{2}H\textsubscript{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\textsubscript{2}H\textsubscript{2}-derived label onto the protein, perhaps through a covalent interaction of the C\textsubscript{2}H\textsubscript{2} with hydrogenase. The mechanism of inhibition of hydrogenase by C\textsubscript{2}H\textsubscript{2} may involve the covalent attachment of C\textsubscript{2}H\textsubscript{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 14C2H\textsubscript{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 (Figure 3) and immunostaining (not shown). The attachment of label from 14C2H\textsubscript{2} to the large subunit leads to an important finding regarding the role of the large subunit in catalysis. Given that C\textsubscript{2}H\textsubscript{2} behaves as an analogue of H\textsubscript{2} and that label from 14C2H\textsubscript{2} is attached only to the large subunit, it follows that the large subunit most likely contains the site of H\textsubscript{2} activation. As such, our experiments provide the first biochemical evidence that the H\textsubscript{2}-activating site is located on the large subunit. This idea is consistent with other observations, as discussed in a recent review (Pryzybyla 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$_2$H$_2$ to hydrogenase which is consistent with the experimental results. To obtain the apparently covalent attachment of C$_2$H$_2$ to hydrogenase, C$_2$H$_2$ must be activated by the enzyme. Given that C$_2$H$_2$ behaves as an analogue of H$_2$, the activation of C$_2$H$_2$ 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$_2$H$_2$, the relatively acidic proton of C$_2$H$_2$ 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$_2$H$_2$ binding to hydrogenase, we have demonstrated the following: (1) C$_2$H$_2$ binds tightly and reversibly to native hydrogenase. (2) Hydrogenase does not catalyze the transformation of C$_2$H$_2$ to another compound. (3) The inhibition is remarkably specific for C$_2$H$_2$. (4) Inhibition of hydrogenase by C$_2$H$_2$ results in the formation of a new protein-staining band which binds C$_2$H$_2$ more strongly and reversibly than native hydrogenase. (5) Denaturation of hydrogenase inhibited with C$_2$H$_2$ reveals the binding of $^{14}$C of the large subunit of hydrogenase, which provides the first biochemical evidence that the H$_2$-activating site of a NiFe hydrogenase is located on the large subunit.

**Registry No.** C$_2$H$_2$, 74-86-2; hydrogenase, 9027-05-8.

**REFERENCES**


