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

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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, binds to and is 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.

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 & Arp, 1986; Kow & 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 1:1 ratio to give a native molecular weight near 100 000. The enzyme also contains Ni and Fe in a 1:10-11 ratio (Seefeldt & Arp, 1986). EPR[1] and UV-vis spectroscopy indicate that the Fe is present in FeS centers, though the exact number and type are not known (Seefeldt, 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

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[1] 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.

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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 & Arp, 1989b), CN^- (Seefeldt & Arp, 1989a), and NO (Hyman & Arp, 1991). This paper deals with the inhibitor C_2H_2. Smith et al. (1976) first recognized the ability of C_2H_2 to inhibit hydrogenase in intact *Azotobacter chroococcum* cells. Yates and co-workers (van der Werf & 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 (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. (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 & 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 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 & 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 & 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 $Na_2S_2O_4$.

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 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* (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 & 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 & 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 (Birkett et al., 1985) with antiserum (200-fold dilution) prepared against *B. japonicum* hydrogenase large subunit or small subunit. Perioxidase-conjugated goat antirabbit antibodies were used diluted 2000-fold (Seefeldt & 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 & Arp, 1990). Briefly, 2.5 mCi of $Ba^{14}CO_3$ (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 $Ba^{14}C_2$ 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 × 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 of water. After 1 h, 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 h, 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 × 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 an activation vial (see *Recovery of Activity following C_2H_2 Inhibition*, above) and was incubated with 101 kPa H_2 for 50 h. 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 & 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 of D_2O (99% purity) to 3 g of CaC_2 in a stoppered side-armed flask (50 mL). The resulting gas was collected in a cryogenic gas purification vessel (Hyman & Arp, 1987b) immersed in liquid N_2. After the hydrolysis of the CaC_2 was complete, the collection vessel was evacuated to remove noncondensed contaminating gases. The collection vessel was then allowed to warm, and the condensed C_2D_2 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 H_2SO_4 trap (Hyman & 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 & Yates, 1978; Hyman & 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 h) 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 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 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 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 (Figure 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,

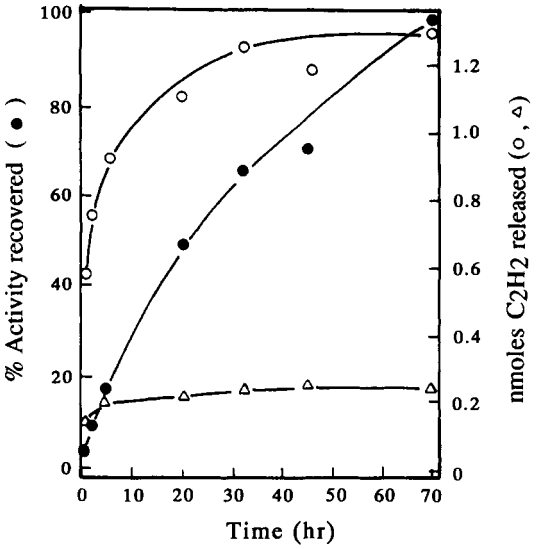

FIGURE 1: Release of C_2H_2 from and recovery of activity by C_2H_2-inhibited hydrogenase. C_2H_2-inhibited hydrogenase (50 μL, 2.54 mg/mL protein) was passed through a Sephdex G-25 column and eluted with 50 mM Tris-HCl (pH 7.5) under Ar to remove the unbound C_2H_2. Eluted fractions which contained 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 (○). 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 (△).

most of the C_2H_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_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 (Figure 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 <1 h). 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 h) 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 h, the activity was inhibited by 67%. Following the removal of the majority of the unbound $^{14}C_2H_2$ from

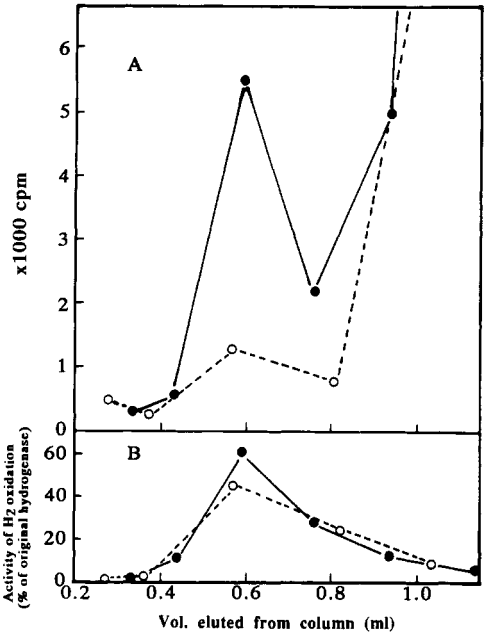

FIGURE 2: Coelution of radioactivity and hydrogenase activity from a gel permeation column following inhibition of hydrogenase with $^{14}C_2H_2$. As described under Materials and Methods, hydrogenase was inhibited with $^{14}C_2H_2$ (●) or $^{14}C_2H_2$ plus H_2 (○) followed by separation of bound and unbound acetylene by passage through a Sephadex G-25 column. Column fractions were analyzed for radioactivity (panel A) and hydrogenase activity (panel B).

the enzyme solution by equilibration with 100 volumes of Ar, 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 $^{14}C_2H_2$ coeluted with hydrogenase activity (Figure 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 was 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 (Figure 1) where the sample taken at the first time point already contained a significant amount of C_2H_2. In the experiment described in Figure 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 bands of radioactivity associated with $^{14}C_2H_2$-inhibited hydrogenase (Figure 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 (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

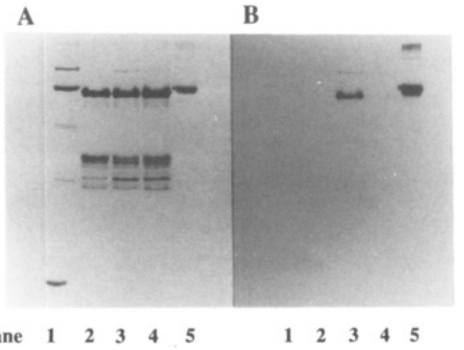

FIGURE 3: SDS–PAGE and fluorography of $^{14}C_2H_2$-inhibited hydrogenase. Hydrogenase samples were inhibited with $^{14}C_2H_2$ with or without H_2 as described under Materials and Methods. Samples (7.1 μg of protein) were then analyzed by SDS–PAGE, and the gels were stained for protein (panel A) and 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).

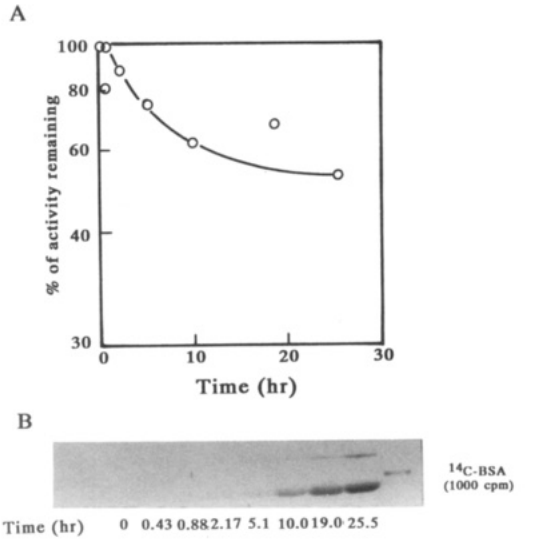

FIGURE 4: Time course of ^{14}C-labeling and inhibition of activity of hydrogenase by $^{14}C_2H_2$. Purified hydrogenase (1.30 mg/mL) was incubated with 4 kPa $^{14}C_2H_2$ and 97 kPa Ar. At the indicated times, a sample (1 μL) was taken to determine hydrogenase activity (panel A), and another sample (10 μL) was taken and mixed with 50 μL of SDS–PAGE sample buffer for further analysis by SDS–PAGE and fluorography (panel B).

(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 (Figure 3) corresponded with a very weak protein-staining band which only appeared in the C_2H_2-treated sample (Figure 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 hydrogenase continues to be inhibited by C_2H_2.

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 and 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 (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 ^{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 Figure 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 $^{14}C_2H_2$, and then activity was allowed to recover following removal of the unbound $^{14}C_2H_2$.

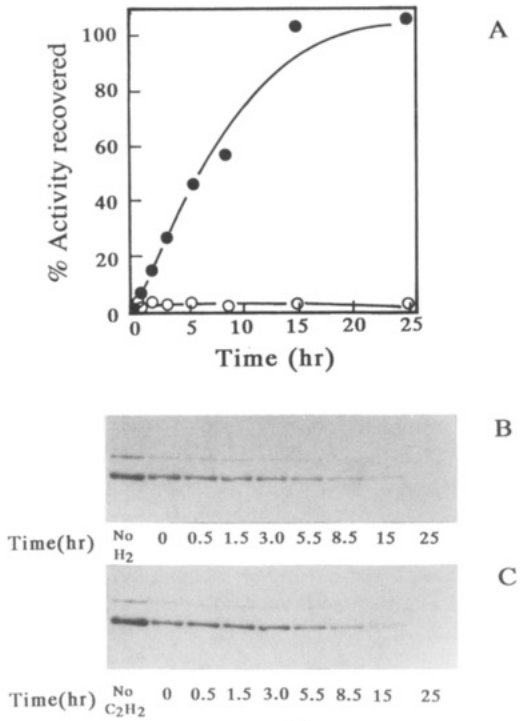

FIGURE 5: Time course of the loss of ^{14}C from and recovery of activity by hydrogenase inhibited with $^{14}C_2H_2$. $^{14}C_2H_2$-inhibited hydrogenase (20 μL, 1.5 mg/mL) was mixed with an anaerobic solution of ovalbumin (80 μL, 1 mg/mL; to serve as a carrier protein) in an Eppendorf tube placed in 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 or 101 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 (○). 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.

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 (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_2H_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 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-h 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 h 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 (Figure 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 (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_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 & 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 described 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 & 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 h) 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 & 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 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

air was sufficient for complete inactivation of a sample not pretreated with C_2H_2 (Seefeldt & Arp, 1989b). The air was then evacuated and replaced with H_2 (101 kPa), and the enzyme was incubated for an additional 52 h (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 & 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 & 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 & Arp, 1987a). (2) Both H_2 activation and C_2H_2 inhibition require catalytically competent enzyme (Hyman et al., 1988). (3) Neither H_2 or 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, 1989). (4) Both H_2 (Seefeldt & 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 & 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 (Figure 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 (Figures 1, 2, and 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 Figures 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 & 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_2H_2 inhibition, C_2H_2 was released from the native enzyme (Figure 1). However, the release of C_2H_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 demonstrated by two independent techniques, namely, measurement by gas chromatography of the C_2H_2 released during the time course of recovery (Figure 1) and determination of the relative amount of ^{14}C label associated with hydrogenase during the recovery (Figure 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

$$\underset{\text{inhibition}}{E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [EI]'} \qquad \underset{\text{recovery}}{[EI]' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E' + I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + I}$$

is the rate constant for conversion of [EI]′ to E′ and k_7 is the rate constant for the conversion of E′ to E. The inhibition phase (formation of [EI]′) was discussed previously (Hyman & Arp, 1987a), and none of the experiments reported here provides 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 (Figure 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 (Figure 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 (Figure 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 (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_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 of 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.

Registry No. C_2H_2, 74-86-2; hydrogenase, 9027-05-8.

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