Rosenthaler, J., Guirard, B. M., Chang, G. W., & Snell, E. E. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 152–158.
Satre, M., & Kennedy, E. P. (1978) *J. Biol. Chem. 253*, 479–483.
Tabor, C. W., & Tabor, H. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 251–282.
Tabor, C. W., & Tabor, H. (1985) *Proc. Int. Congr. Biochem. 13*, 686.
Tanase, S., Guirard, B. M., & Snell, E. E. (1985) *J. Biol. Chem. 260*, 6738–6746.
Williamson, J. M., & Brown, G. M. (1979) *J. Biol. Chem. 254*, 8074–8082.

Acetylene Is an Active-Site-Directed, Slow-Binding, Reversible Inhibitor of *Azotobacter vinelandii* Hydrogenase†

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ABSTRACT: The inhibition of purified and membrane-bound hydrogenase from *Azotobacter vinelandii* by dihydrogen-free acetylene was investigated. The inhibition was a time-dependent process which exhibited first-order kinetics. Both H_2 and CO protected against the inhibition by acetylene. $K_{protect(app)}$ values of 0.41 and 24 μM were derived for these gases, respectively. Both H_2-oxidizing activity and the tritium exchange capacity of the purified enzyme were inhibited at the same rate by acetylene. Removal of acetylene reversed the inhibition for both the purified and the membrane-associated form of the enzyme. The purified hydrogenases from both *Rhizobium japonicum* and *Alcaligenes eutrophus* H16 were also inhibited by acetylene in a time-dependent fashion. These findings suggest that acetylene is an active-site-directed, slow-binding, reversible inhibitor of some membrane-bound hydrogenases from aerobic bacteria.

Acetylene inhibits several microbial gas-utilizing enzymes. Some examples include the concomitant inhibition of both N_2 and proton reduction by nitrogenase (Hwang et al., 1973), the inhibition of N_2O reduction by nitrous oxide reductase (Kristjansson & Hollocher, 1980), and the suicidal inactivation of both the ammonia monooxygenase of the nitrifying bacterium *Nitrosomonas europeae* (Hyman & Wood, 1985) and the methane monooxygenase of the methanotroph *Methylococcus capsulatus* (Bath) (Prior & Dalton, 1985). Detailed kinetic investigations of these inhibitions have provided important insights into the mechanism and catalytic activity of these enzymes. Acetylene has also been reported to inhibit the growth or overall metabolic activities of various hydrogenase-bearing microorganisms. These include methanogenic, sulfate-reducing, and nitrogen-fixing bacteria (Payne, 1984). In these cases, the inhibition caused by acetylene has not been characterized at the enzyme level.

There is considerable confusion in the literature regarding the role of acetylene as an inhibitor of hydrogenases. This confusion is most apparent in studies describing the inhibition of the membrane-bound enzymes found in aerobic, nitrogen-fixing bacteria such as *Azotobacter chroococcum* and *Rhizobium japonicum*. Brotonegoro (1974) first demonstrated that nitrogen-fixing cultures of *A. chroococcum* evolved H_2 after simultaneous treatment with both CO and acetylene. Smith et al. (1976) reappraised these findings and concluded that CO inhibited all nitrogenase reactions other than H_2 production whereas acetylene inhibited the hydrogenase. Subsequently, Walker and Yates (1978) observed that acetylene caused a short-term, irreversible inhibition of hydrogenase activity in continuous cultures of *A. chroococcum*. Furthermore, inhibition of a partially purified hydrogenase from this organism by 40% acetylene could be reversed by flushing with H_2 (Van der Werf & Yates, 1978). In contrast, Laane et al. (1979) reported that 20% acetylene did not inhibit H_2-dependent oxidative phosphorylation in membranes of the related organism *Azotobacter vinelandii*. A similar confusion also exists with the enzyme from *R. japonicum*. For example, Ruiz-Argüeso et al. (1979) failed to inhibit H_2 uptake by soybean bacteroids after treatment with acetylene whereas Arp and Burris (1981) reported an irreversible loss of activity when the purified hydrogenase was incubated for prolonged periods in the presence of acetylene.

In this study, we have considered the role of acetylene as an inhibitor of *A. vinelandii* hydrogenase in both a purified and a membrane-associated form. It should be noted that most commonly available sources of acetylene are often heavily contaminated with H_2 (Hyman & Arp, 1987). The experiments described below have therefore made use of highly purified H_2-free acetylene.

Materials and Methods

Materials

H_2 (99.999%) and N_2 (99.99%) were stripped of residual O_2 by passage over a heated copper-based catalyst (R3-11, Chemical Dynamics Corp., South Plainsfield, NJ). Argon (99.998%) and CO (99.999%) were used without further purification. Gas from an acetylene cylinder (99.6%) was vented until no H_2 was detectable by gas chromatography (detection limit = 2.5 μL/L). After this, the principal contaminants (acetone and phosphine) were removed from the acetylene by passing the gas through a Dreschel bottle containing concen-

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trated sulfuric acid (specific gravity 1.84) and a second bottle containing aqueous 5 M NaOH. The purified gas was then dried by passage through a cartridge containing a commercial desiccant and granular soda-lime (4–8 mesh) (Hyman & Arp, 1987). All cylinder gases were purchased from Liquid Carbonics Corp. (Chicago, IL). Carrier-free $^3H_2$ gas (1 Ci) was obtained from the Lawrence Berkeley Laboratory (Berkeley, CA). Liquid scintillation fluid was purchased from National Diagnostics Inc. (Somerville, NJ).

Methods

Gas Chromatography. H_2 and CO were quantified by using a dual-column Shimadzu gas chromatograph fitted with a thermal conductivity detector. A 5-m stainless-steel column ($^1/_8$th in. o.d.) containing molecular seive 5A was used at a temperature of 100 °C. The detector was operated at 130 °C with a current of 70 mA. Acetylene was quantified by using the same chromatograph described above except a 2-m stainless-steel column ($^1/_8$th in. o.d.) containing Porapak N (80–100 mesh) (Waters Associated, Inc., Milford, MA) was used. All operating conditions were as above. Argon (99.998%) was used as carrier gas at a flow rate of 40 mL/min in both cases.

Protein Purification. The membrane-bound hydrogenase from *A. vinelandii* was purified anaerobically as described previously by Seefeldt and Arp (1986). All enzyme preparations used were highly purified and had specific activities in the range of 55–100 units/mg of protein as determined by a spectrophotometric assay following methylene blue reduction (85 μM) at pH 6.0 and 30 °C in the presence of 101 kPa of H_2. Hydrogenase from *R. japonicum* was purified as described previously (Arp, 1985). The protein used has a specific activity of 60 units/mg of protein. Hydrogenase from *Alcaligenes eutrophus* H16 was purified as described by Arp et al. (1985). After activation in the presence of 2 mM sodium dithionite for 3 h, the enzyme had a specific activity of 20 units/mg of protein.

Preparation of Membranes. Membranes from *A. vinelandii* were stored at −20 °C prior to use. The membranes were prepared for use by thawing and then mixing an equal volume of membranes with buffer [50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)/NaOH, pH 6.0, in this and all subsequent experiments]. This mixture was homogenized in a hand homogenizer and then deoxygenated by repeated evacuation and reequilibration with oxygen-free nitrogen. The membranes were then kept on ice until use. Typically, the membranes had a hydrogenase activity of 0.42 μmol of H_2 oxidized·min^{-1}·(mg of protein)$^{-1}$.

Incubation Procedures. All experiments were carried out under anaerobic conditions. Incubations were carried out in 13-mL serum vials which contained an inner open-topped vessel (total volume of 1 mL) cemented to the serum vial bottom. The serum vials were stoppered with butyl rubber caps and crimped aluminum seals (Wheaton Scientific, Millville, NJ). The required gas phase was added to these stoppered vials after they had been evacuated for 5 min on a vacuum manifold. All incubations were conducted at 1 atm pressure (101 kPa) and 24 °C unless otherwise stated. The inner vial contained the reaction mixture of anaerobic buffer and purified enzyme or membranes. Each 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 all experiments, anaerobic buffer was added to the inner vial and allowed to preequilibrate 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. Unless otherwise stated, reactions of enzyme with inhibitor were initiated by addition of the enzyme to the anaerobic buffer in the inner vial.

Tritium Exchange Assays. Tritium exchange assays were conducted as described previously by Seefeldt et al. (1986).

H_2 Oxidation Assays. All H_2 oxidation assays were conducted as described previously by Arp and Burris (1981) using solutions made anaerobic by sparging with H_2. The following extinction coefficients were used: methylene blue, ϵ = 32.8 mM^{-1}·cm^{-1} at 600 nm; benzyl viologen, ϵ = 8.3 mM^{-1}·cm^{-1} at 600 nm; phenazine methosulfate, ϵ = 26.3 mM^{-1}·cm^{-1} at 387 nm; 2,6-dichloroindophenol, ϵ = 21.0 mM^{-1}·cm^{-1} at 600 nm. All spectrophotometric assays following the absorbance change associated with H_2 oxidation coupled to electron acceptor reduction were linear for at least 30 s. Rates of H_2 oxidation were determined from the initial rates (0–10 s). Absorbance changes were recorded with a Beckman DU-7 spectrophotometer. All kinetic constants were determined by nonlinear least-squares fit to appropriate models (Elsevier Biosoft, Cambridge, England). Estimates of the half-life of enzyme activity under identical conditions were reproducible to within ±9% (for example, see Table I).

Gas Concentrations in Solution. The concentrations of gases in solution were calculated from α values for gas solubility in water at 24 °C. These values were 0.95, 0.02174, and 0.01766 for acetylene, CO, and H_2, respectively (Dean, 1985).

Results

Van der Werf and Yates (1978) reported that 10–40 kPa of acetylene inhibited partially purified hydrogenase from *A. chroococcum* by up to 80% and that this inhibition was only observed when the enzyme was preincubated with the acetylene in the absence of H_2. The approach we adopted was to consider the effect of both the acetylene concentration and the period of exposure to acetylene on the H_2 oxidizing activity of highly purified hydrogenase from *A. vinelandii*. Particular care was made to exclude all H_2 from these experiments.

Our initial experiments revealed that the inhibition was a time-dependent process. Figure 1 shows the loss of activity vs. time for enzyme incubated with a range of acetylene concentrations. With each concentration of acetylene, there was an initial lag phase followed by a time-dependent loss of activity. In the linear portions of these semi-log plots, the inhibition apparently followed a first-order process. The lag phase in the initial parts of these plots may, at least in part, be due to the apparent activation of the enzyme which occurred in the absence of acetylene. However, this activation process cannot fully explain the lag phase because extrapolation of the linear portions of the semi-log plots to zero time results in an estimate of initial activity greater than the final activated rate as measured in the control incubation.

Apparent first-order rate constants (k_{inhib}) for the loss of activity were derived from the data in Figure 1 and plotted as k_{inhib} vs acetylene concentration. The data were fitted to both a straight line and a hyperbola (Figure 1, inset). For practical reasons, we were unable to extend the acetylene concentration range above 303 kPa and therefore were not able to convincingly discriminate between a saturable and a nonsaturable inhibition process. It should be noted that a study of acetylene inhibition using pressures of acetylene in excess of 303 kPa would have to address several complicating factors. These include the very rapid time course of the inhibition process, the potential explosive dangers of pressurized acetylene, and the possibility that secondary denaturing effects would occur when the enzyme was exposed to an organic compound present at concentrations in excess of 0.1 M.

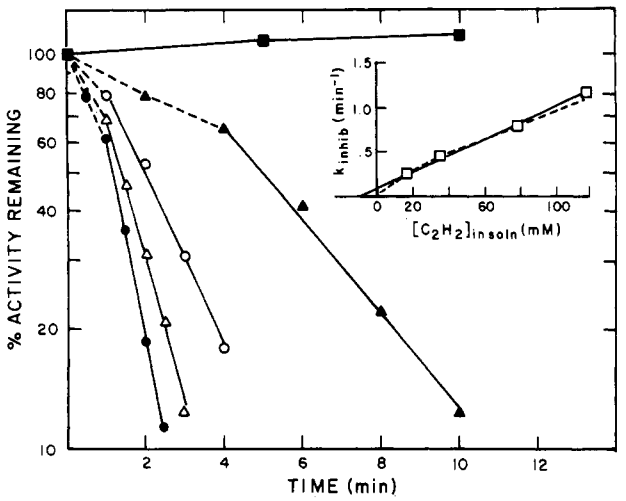

FIGURE 1: Effect of acetylene concentration on the inhibition of purified *Azotobacter vinelandii* hydrogenase. Five incubation vials were prepared as described under Methods. Incubations were initiated by addition of 75 μL (0.15 μg of protein/μL) of enzyme solution to 75 μL of anaerobic buffer. Samples (20 μL) were removed at the indicated times, and their H_2 oxidizing activity was measured with the methylene blue reduction assay. The gas phase in the incubation vials (101 kPa) contained (■) 0, (▲) 47, (○) 90, (△) 202, and (●) 303 kPa of acetylene, and the balance was argon. Apparent first-order rate constants (k_{inhib}) were derived from the data. The inset shows a plot of k_{inhib} vs $[C_2H_2]$.

The significance of conducting the experiments presented in Figure 1 in the complete absence of H_2 is apparent from the results presented in Figure 2. The rate at which inhibition developed was compared for a series of incubations conducted in the presence of 101 kPa of acetylene and increasing concentrations (up to 0.63 kPa) of H_2. As little as 0.21 kPa of H_2 increased the half-life of enzyme activity 3-fold. When the data are replotted as half-life of enzyme activity vs H_2 concentration in solution (Figure 2, inset), the plot is linear, which implies a directly competitive interaction between the binding of acetylene and H_2. The fitted data (r = 0.998) provide an estimate for the apparent $K_{protect}$ of 0.41 ± 0.02 μM for H_2 against acetylene inhibition (Grubmeyer & Gray, 1986).

In the past, many investigators have studied CO as an inhibitor of hydrogenases. In general, CO is a less potent inhibitor of the nickel-containing hydrogenases than the non-nickel-containing enzymes. For example, clostridial hydrogenase (non-nickel) has a K_i for CO of 6 μM (Erbes & Burris, 1978) whereas rhizobial hydrogenase (nickel) has a K_i of 157 μM (Arp & Burris, 1981). Van der Werf and Yates (1978) demonstrated that CO was a competitive inhibitor of H_2 oxidation in *A. chroococcum* with 30 kPa of CO inhibiting H_2 oxidation by 70% in the presence of 10 kPa of H_2. Having demonstrated a competitive effect between H_2 and acetylene, we considered it important to investigate the possible protective effect of CO on the inhibition caused by acetylene.

Figure 3 shows the results of an experiment in which enzyme was incubated under an atmosphere containing both 40 kPa of acetylene and CO concentrations from 0 to 29 kPa. When the observed half-life of H_2 oxidizing activity was plotted vs CO concentration in solution, the plot was linear (Figure 3, inset). The fitted data (r = 0.999) provide an estimate of the apparent $K_{protect}$ for CO protection against acetylene inhibition of 24 ± 7 μM.

A demonstrated protection against acetylene inhibition by CO and H_2, and the known competitive effect of CO with respect to H_2 oxidation, indicates that the binding of all three gases is mutually exclusive and suggests a common binding

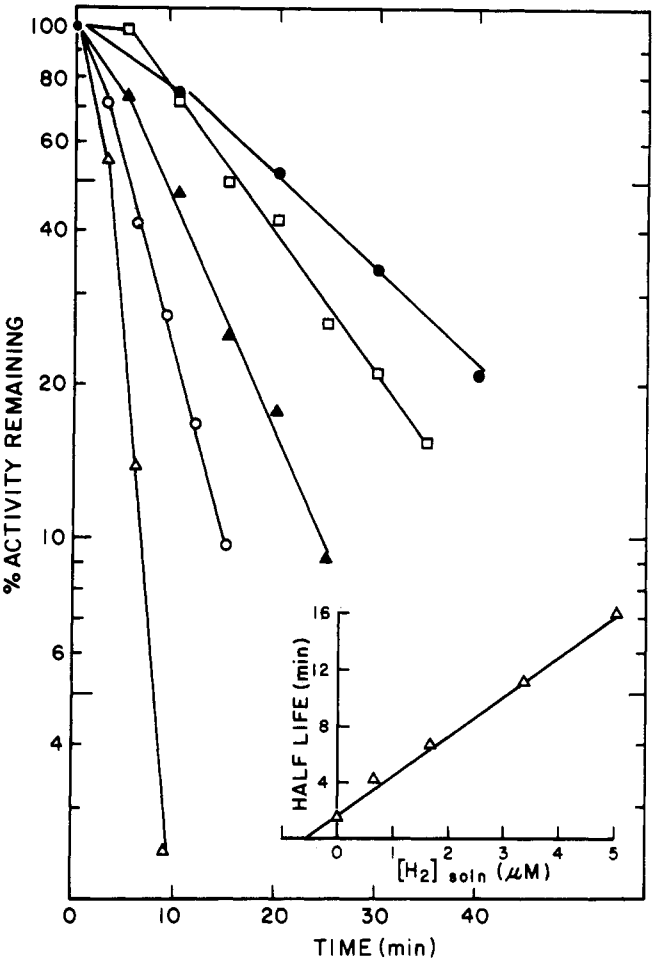

FIGURE 2: Effect of H_2 on the inhibition of purified hydrogenase by acetylene. The gas phase of incubation vials (prepared as described under Methods) consisted of 1 atm (101 kPa) of acetylene and the following H_2 pressures: (△) no addition; (○) 0.08 kPa; (▲) 0.21 kPa; (□) 0.42 kPa; and (●) 0.63 kPa. Each incubation was initiated by the addition of 50 μL (0.34 μg of protein/μL) of purified enzyme solution to 50 μL of buffer in the inner incubation chamber. Samples (10 μL) of the enzyme mixture were removed at the times indicated, and their H_2 oxidizing activity was measured with the methylene blue reduction assay. Half-lives for enzyme activity at each H_2 concentration were derived from the data. The inset shows a plot of half-life of enzyme activity vs H_2 concentration in solution.

Table I: Inhibition of Purified Hydrogenase by Acetylene As Determined with Various Electron Acceptors[a]

electron acceptor	concn (mM) in assay	K_m (mM)	half-life of enzyme act. (min)
benzyl viologen	100	45	14.4
methylene blue	0.085	0.017	15.1
phenazine methosulfate	0.10	0.008	13.8
dichloroindophenol	0.20	ND[b]	15.0

[a] Four incubation vials were prepared as described under Methods. Each vial contained an atmosphere consisting of 20 kPa of acetylene/81 kPa of argon. The incubations were initiated by the addition of 80 μL of enzyme (0.2 mg/mL) to 50 μL of anaerobic buffer in the inner vial. Samples (15 μL) were removed at 15-min intervals, and residual H_2 oxidizing activity was determined by using the electron acceptors listed. The extinction coefficients and assay conditions are described under Methods. [b] Not determined.

site. Furthermore, the binding site for acetylene is not the electron acceptor binding site(s) as indicated by the results in Table I. The degree of inhibition is independent of the electron acceptors we tested, despite their widely varying K_m values.

Isotope exchange reactions have provided a useful probe of

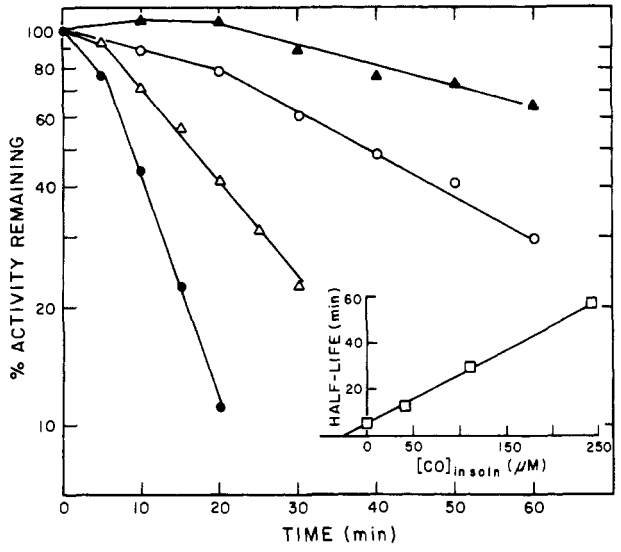

FIGURE 3: Effect of CO on the inhibition of purified hydrogenase by 40 kPa of acetylene. Four incubation vials (prepared as described under Methods) each contained a gas phase (101 kPa) consisting of 40 kPa of acetylene and various concentrations of CO, and the balance was argon. The CO concentrations were 0 (●), 5 (△), 13 (○), and 29 kPa (▲). Each incubation was initiated by the addition of 100 μL (0.15 μg of protein/μL) of enzyme solution to 100 μL of buffer in the inner vial. Samples (20 μL) were removed at regular intervals, and their H_2 oxidizing activity was measured with the methylene blue reduction assay. Half-lives of enzyme activity at each CO concentration were derived from the data. The inset shows a plot of enzyme half-life vs CO concentration in solution.

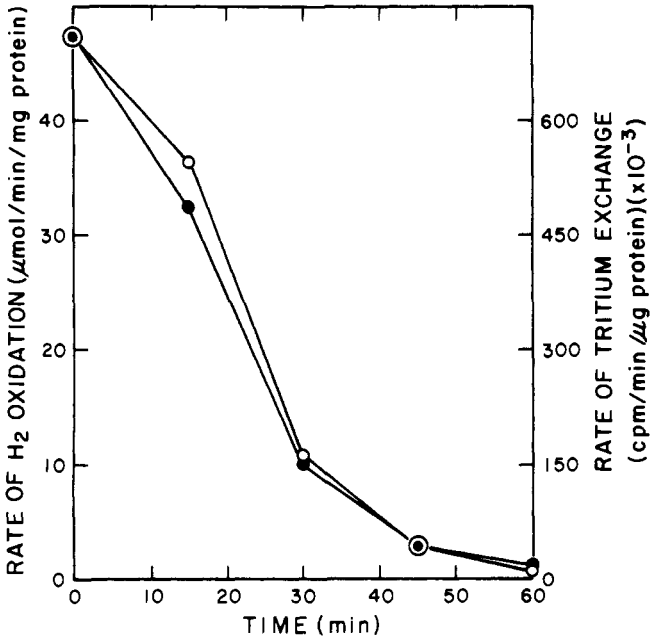

FIGURE 4: Effect of acetylene on the H_2 oxidizing and tritium exchange activity of purified hydrogenase. A single incubation vial was prepared containing 120 μL of anaerobic buffer in the inner vial and an atmosphere consisting of 20 kPa of acetylene/81 kPa of argon. The incubation was initiated by the addition of 60 μL of enzyme (0.2 μg of protein/μL) to the inner vial. Samples were removed at the indicated times and assayed for both residual H_2 oxidizing activity and tritium exchange activity. H_2 oxidizing activity was determined by using the methylene blue reduction assay. Tritium exchange activity was measured as described by Seefeldt et al. (1986). (○) Methylene blue reduction assay. (●) Tritium exchange assay.

the H_2 activating site of hydrogenases. Isotope exchange reactions catalyzed by hydrogenases result in no net change in H_2 concentration of all hydrogen species and are considered to solely involve the H_2 activating site on the enzyme. Figure

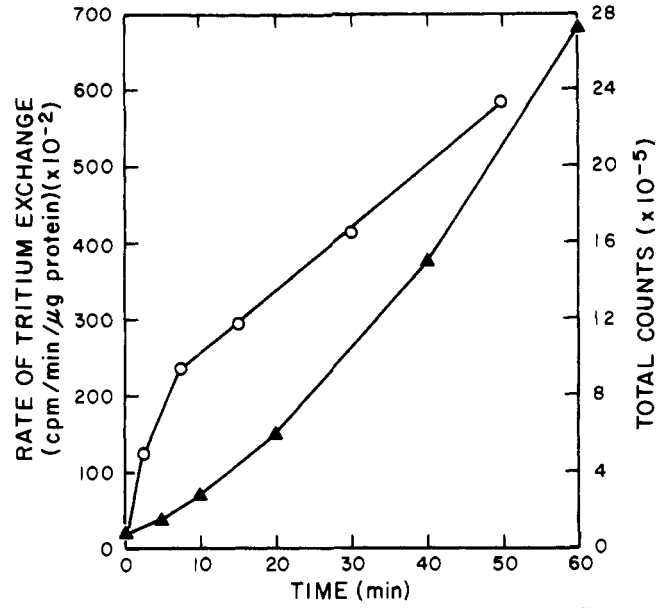

FIGURE 5: Recovery of tritium exchange activity of purified hydrogenase after inhibition by 20 kPa of acetylene. Sixteen microliters of an enzyme solution exposed to 20 kPa of acetylene/80 kPa of argon for 1 h (see Figure 4) was added to a tritium exchange assay vial. Samples (10 μL) were removed from this vial at the times indicated, and the total counts from a 1% aliquot were determined as described by Seefeldt et al. (1986): (▲) total counts; (○) rate of tritium exchange expressed as cpm per microgram of protein. Rates of tritium exchange are plotted as midpoints between the time points used for their determination.

4 shows that H_2 oxidation and the tritium exchange capability were lost at the same rate when the enzyme was exposed to acetylene. The concurrent loss of these activities further supports a direct interaction between acetylene and the H_2 activating site of the enzyme.

In the course of running several tritium exchange experiments, it was noted that the rate of tritium exchange increased with the length of time acetylene-inhibited enzyme was left in the tritium exchange reaction vessel. A typical example of this is shown in Figure 5. This figure shows that the rate of increase in the rate of tritium exchange initially approximates to a hyperbola but then deteriorates to a linear regain of activity. A similar, nonlinear pattern for the recovery of activity was observed when an incubation vial containing fully inhibited enzyme was flushed with either H_2 or argon. With a humidified gas venting rate of 750 mL/min, the enzyme recovered 20% and 22% of its original, preinhibited activity after 3 h venting with H_2 and argon, respectively. This result shows H_2 itself does not promote or stimulate the recovery of activity of acetylene-inhibited enzyme.

In the studies made by Yates and co-workers, the principal difference noted between the inhibition of partially purified enzyme and that of whole cells was that the inhibition caused by acetylene was essentially irreversible in continuous cultures of *A. chroococcum* and reversible in the purified enzyme. These findings suggest that the purification process might affect the response of the enzyme to inhibition by acetylene. Certainly, it is known that the purification of rhizobial hydrogenase from membranes results in a 20-fold increase in the K_m for H_2 from 50 nM to 1 μM (Emerich et al., 1980; Arp & Burris, 1981). Several of the experiments described above for the purified hydrogenase were, therefore, repeated with membranes. When membranes were incubated under H_2-free conditions in the presence of various concentrations of acetylene, semilog plots of percent remaining activity vs time were linear, after an initial lag phase. The half-lives of activity of

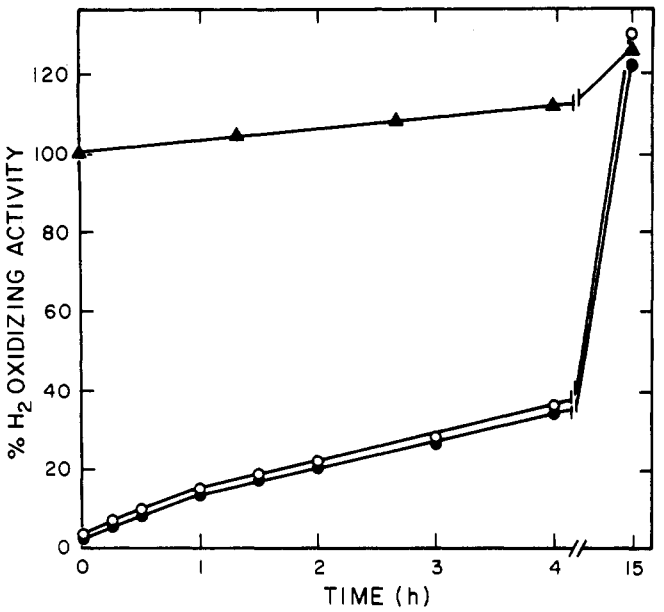

FIGURE 6: Reversibility of inhibition of hydrogenase by acetylene in membranes of *Azotobacter vinelandii*. Three incubation serum vials were prepared containing 100 μL of anaerobic buffer in the inner vial. The vials also contained the following initial gas phases (101 kPa): (▲) 101 kPa of argon; (○) 20 kPa of acetylene/81 kPa of argon; (●) 80 kPa of acetylene/21 kPa of argon. Homogenized anaerobic membranes (100 μL) were then added to the inner vial to give a final protein concentration of 16.5 mg/mL. After 1 h, 20-μL samples were removed, and their H_2 oxidizing activity was measured with the methylene blue reduction assay. O_2-free, humidified H_2 was then flowed through each vial at a rate of 160 mL/min. Samples (10 μL) removed at regular intervals over a 4-h period were assayed for H_2 oxidizing activity. A final sample was removed 15 h after the initiation of the H_2 flow.

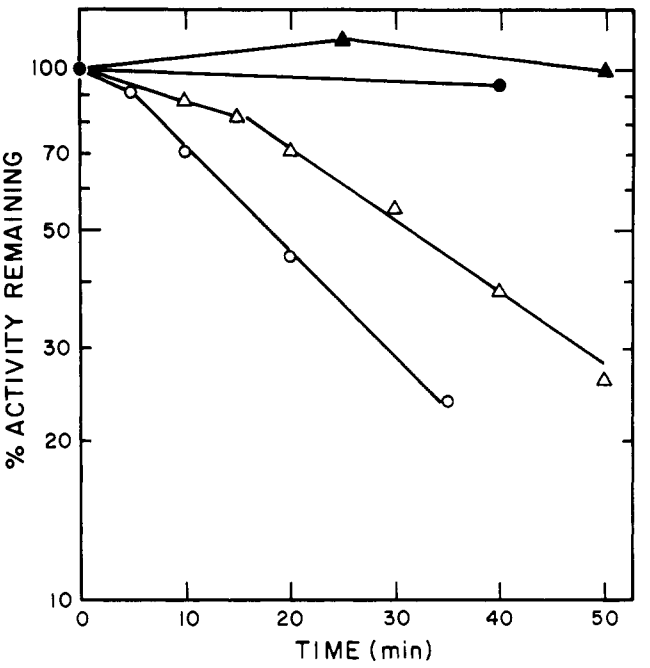

FIGURE 7: Inhibition of purified, membrane-bound hydrogenases from *Rhizobium japonicum* and *Alcaligenes eutrophus* H16 by acetylene. Two sets of incubation serum vials were prepared which contained 1 atm of either acetylene or argon. Incubations using *A. eutrophus* hydrogenase were initiated by the addition of 250 μL of activated enzyme (0.24 μg of protein/μL) to 360 μL of anaerobic buffer in the inner vial. Incubations using *R. japonicum* hydrogenase were initiated by the addition of 75 μL of enzyme (1.77 μg of protein/μL) to 75 μL of anaerobic buffer in the inner vial. Samples were removed at the indicated times, and their H_2 oxidizing activity was measured by using the methylene blue reduction assay. Sample sizes were 20 μL for *A. eutrophus* and 5 μL for *R. japonicum* experiments. The figure shows a semi-log plot of remaining activity vs time for *A. eutrophus* H16 hydrogenase incubated under (●) 101 kPa of argon and (○) 101 kPa of acetylene and for *R. japonicum* hydrogenase incubated under (▲) 101 kPa of argon and (△) 101 kPa of acetylene.

membranes incubated under atmospheres of 20, 30, 50, and 80 kPa of acetylene in argon were 7.2, 4.5, 2.6, and 1.8 min, respectively.

Similarities between the purified and membrane-associated enzyme were also observed in the protective effect of H_2 and CO provided against acetylene inhibition. As little as 0.04 kPa of H_2 resulted in a 3-fold increase in activity half-life when membranes were incubated under 25 kPa of acetylene. Likewise, 75 kPa of CO fully protected membranes from inhibition by 10 kPa of acetylene over a 30-min period.

Our final observation outlining the similar response of purified and membrane-associated enzyme to acetylene is shown in Figure 6. In this experiment, acetylene-treated membranes were flushed continuously with H_2 to remove acetylene from the incubation vial. Methylene blue reduction assays conducted at regular intervals revealed that there was a nearly linear return of activity to the membranes. No difference was noted in the rate of return of activity for membranes inhibited by either 20 or 80 kPa of acetylene. Full activity could be regained after 15 h flushing with H_2. A similar experiment to that described in Figure 6 was conducted over a shorter time scale using membranes fully inhibited with 50 kPa of acetylene and then continuously flushed with either argon, H_2, or CO. In all cases, the activity was returned at the same constant rate and was equivalent to 22–25% of the preinhibited rate after 3 h (data not shown).

Having characterized the inhibition of the hydrogenase from *A. vinelandii* by acetylene, we considered whether similar effects were obtainable with other hydrogenases from aerobic organisms. Figure 7 shows that the purified hydrogenases from both *R. japonicum* and *A. eutrophus* H16 were inhibited in a time-dependent fashion by acetylene. Distinct differences are apparent in the susceptibility of these enzymes to acetylene. The half-lives for *Rhizobium* and *Alcaligenes* hydrogenases were 20.8 and 15.7 min, respectively, under 101 kPa of C_2H_2.

Discussion

The results of the experiments described in this paper can be summarized by the following three points: First, the inhibition of *A. vinelandii* hydrogenase by acetylene is a time-dependent process which exhibits first-order kinetics. The rate of inhibition is dependent upon the acetylene concentration over the range of concentrations tested. Second, the inhibition caused by acetylene is apparently directed toward the H_2 activating site of hydrogenase because (a) both H_2 and CO competitively protect against the inhibition by acetylene, (b) the rate of H_2 oxidizing activity is inhibited by acetylene at the same rate as the tritium exchange capacity of the enzyme, and (c) the inhibition occurs in the absence of any electron acceptor (and, therefore, net electron flow) and is independent of the electron acceptor used to support activity. Third, the inhibition is reversible, albeit slowly, in both the purified and the membrane-associated forms of the enzyme. All of these characteristics are compatible with acetylene acting as a slow and tight-binding inhibitor of *A. vinelandii* hydrogenase. As a group of inhibitors, slow-binding and slow, tight-binding inhibitors are characterized by a slow rate of establishment of equilibrium between enzyme, inhibitor, and enzyme–inhibitor complex (Morrison, 1982). There are two generally accepted kinetic mechanisms which account for the effects observed with this form of inhibitor.

mechanism 1

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

mechanism 2

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (EI)^*$$

Both mechanisms predict a first-order loss of activity when enzyme is incubated with excess inhibitor. Mechanism 1 involves a slow but direct conversion of free enzyme to an inactivate EI complex, whereas mechanism 2 involves an initial rapid conversion of enzyme to a transient EI complex, followed by a slow conversion of EI to EI*. Mechanisms 1 and 2 can be discriminated by two main kinetic considerations. For mechanism 1, there is a linear relationship between the rate of inhibition (k_{inhib}) and the inhibitor concentration. Furthermore, the presence of the inhibitor has no effect on the initial rate of reaction when enzyme is incubated with both inhibitor and substrate. With mechanism 2, the relationship between k_{inhib} and inhibitor concentration is hyperbolic, indicating the inhibition is a saturable process. In addition, the presence of inhibitor decreases the initial rate of reaction in proportion to inhibitor concentration (Morrison, 1982). The use of progress curves to study slow-binding inhibition allows for a clear discrimination between mechanisms 1 and 2 and provides a direct estimate of the K_i for the inhibitor under investigation. In the case of hydrogenase, the time course of acetylene inhibition is so slow with respect to substrate turnover, and the substrate itself is such a potent protecting agent against acetylene inhibition, that progress curves are an inappropriate means of studying acetylene inhibition.

The alternative to considering the effects of acetylene on progress curves is to consider the relationship between k_{inhib} and inhibitor concentration, as shown in Figure 1. The inset in Figure 1 shows that a plot of k_{inhib} vs acetylene concentration can be fitted satisfactorily by least-squares fit to both a straight line and a hyperbola. In the case of the straight line fit (r = 0.995), the plot shows a nonzero intercept which implies a deviation from simple adherence to mechanism 1. In the case of the hyperbolic fit (r = 0.997), the data provide an estimate of the maximal rate of inhibition (k_3 in mechanism 2) of 3.8 ± 1.1 min^{-1} and a $K_{i(app)}$ value of 270 ± 104 mM. The same analysis of the data obtained with the inhibition of the hydrogenase in membranes provides a straight line fit with a correlation coefficient of 0.999 and a fit to a hyperbola (r = 0.999) which provides an estimate for k_3 of 3.7 ± 0.4 min^{-1} and an estimate for $K_{i(app)}$ of 320 ± 41 mM.

It is clear from these data that we cannot discriminate unequivocally between mechanisms 1 and 2. In this study (data not shown) and a previous study (Arp & Burris, 1981), acetylene had no effect on the initial rate of H_2 oxidation at low H_2 concentrations, an observation that supports mechanism 1. However, it is not unreasonable to suggest that acetylene inhibition is in fact a saturable phenomenon at sufficiently high acetylene concentrations. The principal limitation of the present evidence supporting mechanism 2 is that the concentrations of acetylene used do not even approach the estimated value of $K_{i(app)}$. However, to reach these concentrations would require a minimum acetylene gas pressure in excess of 600 kPa. The practical considerations limiting the range of acetylene concentrations have been discussed earlier.

While mechanistic considerations can alter the validity of estimates of the $K_{i(app)}$ for acetylene, the apparent values of $K_{protect}$ we have derived for both H_2 (0.41 ± 0.02 μM) and CO (24 ± 7 μM) are independent of inhibitor mechanism. The values of $K_{protect(app)}$ require modification according to eq 1:

$$K_{protect(true)} = K_{protect(app)}(1 + [C_2H_2]/K_{i(app)}) \quad (1)$$

When a tentative estimate of the $K_{i(app)}$ for acetylene of 300 mM is used, the values of $K_{protect(app)}$ and $K_{protect(true)}$ differ by 14% or less. The true values of $K_{protect}$ can then be estimated at 0.37 ± 0.02 μM for H_2 and 23 ± 6 μM for CO. The experiments from which these constants were derived used enzyme incubated in the absence of electron acceptor and hence substrate turnover. As these experiments only consider the effects of H_2 or CO binding on the rate of acetylene inhibition, the values of $K_{protect(true)}$ can be equated with the true binding constants (K_D) for these gases. These values can then be compared with the estimates of K_m for H_2 for this hydrogenase of between 0.86 μM (Seefeldt & Arp, 1986) and 1–2 μM (Kow & Burris, 1984) and a K_i for the reversible competitive inhibition of H_2 oxidation by CO of 25 μM (D. J. Arp et al., unpublished results).

In contrast to the problems discussed above in relation to the forward rate of inhibition (k_1 in mechanism 1 and k_3 in mechanism 2), the reverse rate constant (k_2 in mechanism 1 and k_4 in mechanism 2) is independent of substrate, enzyme, and inhibitor concentration (Morrison, 1982). Under ideal circumstances, the limiting reverse rate constant can be estimated directly from the expected exponential recovery of activity when fully inhibited enzyme is allowed to dissociate from the inhibitor under inhibitor-free conditions. We have considered the recovery of activity of acetylene-inhibited hydrogenase using two independent methods. The results presented in Figure 5 obtained with tritium exchange assays showed that the enzyme recovered activity in an initially exponential fashion which declined to a zero-order process with time. A similar recovery of activity was also observed in other experiments (e.g., Figure 6) which monitored activity using the less sensitive methylene blue reduction assay. Clearly, a near-linear return of activity of the hydrogenase is a departure from the behavior expected with this form of inhibition. However, similar zero-order recoveries of activity have been observed in tight-binding inhibition studies which have utilized far more stable enzymes than the solubilized hydrogenase used in the present study (Cha et al., 1975; Badet et al., 1986). We can discount the possibility that there is a diffusion limitation on the removal of acetylene because the results in Figure 6 showed that there was no difference in the rate of recovery of activity of membranes fully inhibited with 20 or 80 kPa acetylene. It would, therefore, appear that some factor other than inhibitor release is limiting the rate of recovery of activity. Considering the length of time over which the recovery of activity occurs, any number of explanations, including lability of the enzyme, must be considered. In view of this complication, the return rate constant, k_4, can only be estimated indirectly. The most accurate estimate of k_4 can be made from the results presented in Figure 5. If an exponential recovery of activity were continued during the experiment, 12% of the preinhibited activity would be recovered within 1 h. This value enables us to extrapolate a half-life for recovery of activity of 330 min, resulting in an estimate for k_4 (mechanism 2) of 0.0021 min^{-1}.

The two main points we have considered in describing acetylene as a slow-binding inhibitor are the slow, time-dependent nature of the inhibition and the even slower return of activity upon removal of excess inhibitor. The data we have presented demonstrating these effects suggest there is a considerable barrier to the inhibitory binding of acetylene to the hydrogenase. One element of this barrier probably involves

a structural limitation where acetylene has limited access to an active site adapted to the small hydrogen molecule. On the other hand, this barrier may also represent a more complex process which involves a step in which a correctly oriented acetylene molecule is effectively "trapped" by the enzyme during a process normally required for substrate turnover. Morrison (1982) has suggested that slow-binding inhibition may arise from transitional conformational changes associated with catalysis which are induced by the inhibitor. The slowly reversible nature of this form of inhibition then arises due to a restriction on the release of the "clamped" inhibitor and the inability of the enzyme to undergo the conformational changes associated with product release. It may also be that hydrogenase interacts more actively with acetylene during the slow step, resulting in a chemical change in the acetylene. For example, a proton might be abstracted from acetylene, leaving an acetylide bound to the active site. This would be analogous to the presumed mechanism of H_2 splitting, where removal of a proton results in a hydride bound to the active site (Krasna, 1979).

Further clarification of the mechanism of acetylene inhibition of this enzyme may be forthcoming from a study of the lag phase which is apparent in the semi-log plots presented in Figures 1–3. The reasons for this lag are not readily apparent. We consider it unlikely to reflect a diffusion limitation of acetylene into the enzyme solution given that the lag is apparent for up to 20 min in certain circumstances (see Figure 3). We also consider it unlikely that the lag phase is due to a transformation of acetylene to an alternative, more potent inhibitor. For example, ethylene, a possible product of an enzymatic transformation of acetylene, has no effect on the activity of this enzyme over a 1-h period (data not shown). It should be noted that the presence of a lag phase in the time-dependent loss of activity is a process not accounted for in the present models (mechanisms 1 and 2) commonly used to describe slow-binding inhibition. It may be that our inability to clearly discriminate between mechanisms 1 and 2, the presence of a lag phase, and the apparent deviation observed in the recovery of activity of fully inhibited enzyme shown in this study point to the existence of a more complex process in acetylene inhibition which is not accounted for by the present models.

Beyond providing a preliminary characterization of the kinetics of the inhibition of this hydrogenase by acetylene, the results presented in this study also provide a possible explanation for some of the confusion which has surrounded this problem in the past. The most significant point is that hydrogen is a very potent protecting agent against acetylene inhibition. Prior to this study, we had observed that both cylinder acetylene and acetylene generated in the laboratory using calcium carbide are often highly contaminated with H_2 (Hyman & Arp, 1987). The results presented in Figure 2 show that even very low H_2 concentrations can dramatically alter the apparent inhibition pattern. This effect would explain why Laane et al. (1979) failed to detect an inhibition of hydrogenase activity when *A. vinelandii* membranes were incubated under an atmosphere containing 20% acetylene and 50% H_2. Addition of H_2 either intentionally or as a contaminant in acetylene would explain why other investigations have failed to detect acetylene inhibition of hydrogenase. On the other hand, the results in Figure 7 show that hydrogenases which have been shown to be physiologically, structurally, and immunologically related (Arp et al., 1985) do show distinct differences in their susceptibilities to acetylene even in the strict absence of H_2. Care should therefore be taken when extrapolating the results presented in this paper from one organism to another.

The clarification of the role of acetylene as an inhibitor of hydrogenases from aerobic, nitrogen-fixing bacteria now provides a powerful tool in the study of the mechanism of these enzymes. Recently, the inhibition of the hydrogenase from *Chromatium vinosum* by CO was studied by using electron paramagnetic resonance spectroscopy. A direct interaction between carbon monoxide and the nickel present in this enzyme is suggested by this work (van der Zwaan et al., 1986). A similar study using acetylene as an active-site-directed inhibitor could provide important insights into the possible role of nickel in the catalytic activity of nickel-containing hydrogenases.

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REFERENCES

Arp, D. J. (1985) *Arch. Biochem. Biophys. 237*, 504–512.
Arp, D. J., & Burris, R. H. (1981) *Biochemistry 20*, 2234–2240.
Arp, D. J., McCollum, L. C., & Seefeldt, L. C. (1985) *J. Bacteriol. 163*, 15–20.
Badet, B., Inagaki, K., Soda, K., & Walsh, C. T. (1986) *Biochemistry 25*, 3275–3282.
Brotonegoro, S. (1974) Thesis, Mededelingen Landbouwhogschool, Wageningen, The Netherlands.
Cha, S., Agarwal, R. P., & Parks, R. E. (1975) *Biochem. Pharmacol. 24*, 2187–2197.
Dean, J. A., Ed. (1985) *Lange's Handbook of Chemistry*, 13th ed., pp 10/3–10/5, McGraw-Hill, New York.
Emerich, D. W., Ruiz-Argüeso, T., Russell, S. A., & Evans, H. J. (1980) *Plant Physiol. 66*, 1061–1066.
Erbes, D., & Burris, R. H. (1978) *Biochim. Biophys. Acta 525*, 45–54.
Grubmeyer, C. T., & Gray, W. R. (1986) *Biochemistry 25*, 4778–4784.
Hwang, J. C., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292*, 256–270.
Hyman, M. R., & Wood, P. M. (1985) *Biochem. J. 227*, 719–725.
Hyman, M. R., & Arp, D. J. (1987) *Appl. Environ. Microbiol. 53*, 298–303.
Kow, Y. W., & Burris, R. H. (1984) *J. Bacteriol. 159*, 564–569.
Krasna, A. I. (1979) *Enzyme Microb. Technol. 1*, 165–172.
Kristjansson, J. K., & Hollocher, T. C. (1980) *J. Biol. Chem. 255*, 704–707.
Laane, C., Haaker, H., & Veeger, C. (1979) *Eur. J. Biochem. 97*, 369–377.
Morrison, J. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 102–105.
Payne, W. J. (1984) *J. Microbiol. Methods 2*, 117–133.
Prior, S. D., & Dalton, H. (1985) *FEMS Microbiol. Lett. 29*, 105–109.
Ruiz-Argüeso, T., Emerich, D. W., & Evans, H. J. (1979) *Arch. Microbiol. 121*, 199–206.
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.
Smith, L. A., Hill, S., & Yates, M. G. (1976) *Science (Washington, D.C.) 262*, 209–210.

Van der Werf, A. N., & Yates, M. G. (1978) in *Hydrogenases: Their Catalytic Activity, Structure and Function* (Schlegel, H. G., & Schneider, K., Eds.) pp 307–326, Erich Goltze, K. B., Göttingen, FR.

van der Zwaan, J. W., Albracht, S. P. J., Fontijn, R. D., & Roelofs, Y. B. M. (1986) *Biochim. Biophys. Acta 862*, 208–215.

Walker, C. C., & Yates, M. G. (1978) *Biochimie 60*, 225–231.

Contribution of 3-O- and 6-O-Sulfated Glucosamine Residues in the Heparin-Induced Conformational Change in Antithrombin III†

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ABSTRACT: The role of 3-O- and 6-O-sulfated glucosamine residues within the heparin octasaccharide critical for biological activity, iduronic acid[(1)]→*N*-acetylglucosamine 6-*O*-sulfate[(2)]→glucuronic acid[(3)]→N-sulfated glucosamine 3,6-di-*O*-sulfate[(4)]→iduronic acid 2-*O*-sulfate[(5)]→N-sulfated glucosamine 6-*O*-sulfate[(6)]→iduronic acid 2-*O*-sulfate[(7)]→anhydromannitol 6-*O*-sulfate[(8)], was determined by comparing its ability to bind antithrombin, induce a conformational change in this protease inhibitor as monitored by the enhancement of intrinsic fluorescence, and accelerate (*at saturation*) the interaction of this protein with human factor Xa. The octasaccharide produced a maximum 48% increase in intrinsic fluorescence at 37 °C and a rate of factor Xa inhibition of 6×10^5 M^{-1} s^{-1} as measured by stopped-flow fluorometry at 25 °C. The basal rate of the antithrombin–factor Xa interaction observed in the absence of oligosaccharide was 2×10^3 M^{-1} s^{-1}. The synthetic pentasaccharide, consisting of residues 2–6, produced fluorescence enhancement and rate of inhibition equal to those of the octasaccharide. However, a similar pentasaccharide, identical in all respects except that it lacked the 3-*O*-sulfate on residue 4, produced less than a 5% fluorescence enhancement and a rate of factor Xa inhibition of 8×10^3 M^{-1} s^{-1}. The tetrasaccharide consisting of residues 2–5 produced a 35% fluorescence enhancement and a rate of factor Xa inhibition of 3×10^5 M^{-1} s^{-1}. The tetrasaccharide consisting of residues 3–6 produced a 33% fluorescence enhancement and a rate of factor Xa inhibition of 6×10^4 M^{-1} s^{-1}. Thus, the loss of either the 6-O-sulfated residue 2 or the 3-*O*-sulfate of residue 4 results in a 30–95% loss in the ability of the pentasaccharide to enhance the intrinsic fluorescence of antithrombin and a 10–76-fold reduction in the rate of factor Xa inhibition. These two residues must, necessarily, represent the major contributors to a conformational change in antithrombin, which is linked to the biological activity of the octasaccharide. In addition, nonproportional changes in the intrinsic fluorescence enhancement and the acceleration of factor Xa neutralization indicate that multiple conformational stages can occur in antithrombin when complexed to these oligosaccharides.

Heparin (H) is a highly sulfated polysaccharide that functions by binding to antithrombin (AT) and accelerating the rate at which this protease inhibitor neutralizes proteolytic enzymes of the hemostatic mechanism (Rosenberg, 1977). Only a small fraction of all heparin preparations exhibits high affinity for antithrombin and is responsible for virtually all of the anticoagulant properties of the polysaccharide (Lam et al., 1976). This fraction of anticoagulantly active heparin was found to contain an antithrombin binding domain with the unique tetrasaccharide sequence iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 6-*O*-sulfate (Rosenberg et al., 1978; Rosenberg & Lam, 1979; Lindahl et al., 1979). Subsequently, Leder (1980) isolated a sulfatase that specifically removes 3-*O*-sulfate groups from nonreducing end glucosamine residues of heparin and postulated that this unique substituent is present within the antithrombin binding domain of heparin. Data provided by Lindahl et al. (1980) and Casu et al. (1981) confirmed this supposition and located the 3-*O*-sulfate on the glucosamine moiety at the reducing end of the unique tetrasaccharide sequence. Choay et al. (1980), Casu et al. (1981), Oosta et al. (1981), Riesenfeld et al. (1981), and Atha et al. (1984b) have isolated octasaccharide fragments from deaminative as well as enzymatic cleavage products of heparin that contain this tetrasaccharide binding region. These oligosaccharides exhibit a high affinity for antithrombin as well as the capacity to accelerate the formation of the factor Xa–protease inhibitor complex. Studies by Choay et al. (1981) suggested that the major features of the antithrombin binding site of heparin are

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