

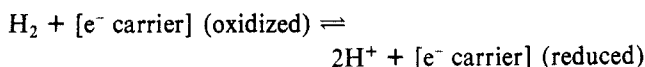
Kinetic Mechanism of the Hydrogen-Oxidizing Hydrogenase from Soybean Nodule Bacteroids[†]

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ABSTRACT: The kinetic mechanism of the unidirectional H₂-oxidizing hydrogenase from soybean nodule bacteroids has been investigated with highly purified enzyme. Measurements of the *K_m* for H₂ vary from 0.97 to 2.6 μM, and the *K_m* for methylene blue varies from 6 to 17 μM. With H₂ and methylene blue as substrates, the initial velocity patterns are intersecting. High levels of methylene blue are inhibitory (*K_i* = 2.4 mM): the inhibition is competitive vs. H₂. CO is a competitive inhibitor of H₂ (*K_i* = 157 μM) and noncompetitive vs. methylene blue. O₂ inactivates the enzyme (*t*_{1/2} ~ 1 h) but also is a reversible inhibitor of hydrogenase activity upon short exposure to low concentrations. Inhibition by O₂ is uncom-

petitive vs. H₂ and noncompetitive vs. methylene blue. Hydrogenase was not inhibited by C₂H₂; preincubation under C₂H₂ inactivates the enzyme. Reduced methyl and benzyl viologens support low rates of H₂ evolution by the hydrogenase. The *K_m* for reduced methyl viologen is 11 μM. H₂ is a potent inhibitor of H₂ evolution: the inhibition is noncompetitive vs. reduced methyl viologen. The hydrogenase will catalyze a low rate of exchange in the reaction between D₂ and H₂O to form both HD and H₂. We propose a two-site ping-pong mechanism for the enzyme in which H₂ is reversibly activated at one site and e⁻ carriers interact at the second site.

Hydrogenase is an iron-sulfur protein that catalyzes the activation of molecular hydrogen. This activation may result in oxidation or evolution of H₂:



Hydrogenases have been isolated from a wide variety of microorganisms and may be bidirectional or unidirectional. Bidirectional hydrogenases are capable of H₂ oxidation and evolution in vitro, and their in vivo function depends on the cell type and growth conditions. Unidirectional hydrogenases oxidize H₂ in vivo and have been referred to as "uptake hydrogenases". In vitro, the unidirectional hydrogenases usually can be made to evolve H₂, but the rates of evolution are low (0-3%) relative to the rate of H₂ oxidation (Arp & Burris, 1979; Van der Werf & Yates, 1978; Chen & Blanchard, 1978; Houchins, 1980). Several hydrogenases have been purified and characterized with regard to their molecular weight, subunit structure, Fe content, and other properties (Mortenson & Chen, 1974; Schlegel & Schneider, 1978).

Detailed kinetic studies of hydrogenase have been limited to bidirectional hydrogenases. Studies of H₂-D₂O exchange and parahydrogen to orthohydrogen conversion in *Clostridium pasteurianum*, *Proteus vulgaris*, and *Desulfovibrio vulgaris* have indicated a heterolytic split of H₂ to form a proton and a hydride as intermediates (Krasna & Rittenberg, 1954; Tamiya & Miller, 1963; Yagi et al., 1973). The kinetics of methyl viologen oxidation and reduction by clostridial hydrogenase (Erbes & Burris, 1978) and the steady-state kinetics of *Thiocapsa roseopersicina* hydrogenase (Varfolomeev et al., 1978) were studied and used to deduce kinetic mechanisms for those enzymes. In this paper, we report the kinetic mechanism of the unidirectional H₂-oxidizing hydrogenase

from soybean root nodules. This hydrogenase is of interest as it serves in scavenging the H₂ generated in situ by nitrogenase.

Materials and Methods

Chemicals. Methyl viologen, 2,6-dichlorophenolindophenol, methylene blue, phenazine methosulfate, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were obtained from Sigma Chemical Co., benzyl viologen was from Mann Research Labs, potassium ferricyanide was from Mallinckrodt, and sodium dithionite was from J. T. Baker Co. CO and D₂ were obtained from Matheson, H₂, O₂, and argon were high-purity commercial grade, and C₂H₂ was generated by adding CaC₂ (Sargent-Welch) to water.

Plant Growth and Enzyme Purification. Hodgson soybean seeds inoculated with *Rhizobium japonicum* 110 were grown and harvested as described (Arp & Burris, 1979). Hydrogenase was purified (Arp & Burris, 1979) from bacteroids isolated from soybean nodules. The purification included detergent solubilization of the enzyme from the bacteroid membrane, poly(ethylene glycol) fractionation, and ion-exchange and gel filtration chromatography. All steps were performed anaerobically, and the enzyme was stored anaerobically at 4 °C.

Methods. Hydrogenase activity was monitored either spectrophotometrically or amperometrically. In the spectrophotometric technique, reduction of an e⁻ carrier was monitored in double-serum-stoppered cuvettes (Figure 1) with a 2-mL liquid phase and a 8-mL gas phase adjusted as indicated. Extinction coefficients for methyl viologen, dichlorophenolindophenol, ferricyanide, and phenazine methosulfate were accepted from the literature. The extinction coefficient for benzyl viologen at pH 6.0 was determined to be 8.3 cm⁻¹ mM⁻¹ at 600 nm. The extinction coefficient at 600 nm for methylene blue varied with concentration. From 0 to 20 μM, a value of 22.4 cm⁻¹ mM⁻¹ was used and from 50 to 100 μM a value of 32.8 cm⁻¹ mM⁻¹. With the amperometric technique, the H₂ concentration was measured directly with a H₂ electrode (Sweet et al., 1980; Wang et al., 1971). Reactions were run in a water-jacketed chamber equipped with an O₂ and a H₂

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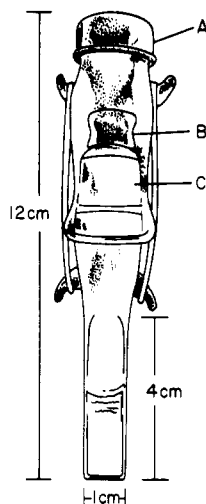


FIGURE 1: Double-serum-stoppered cuvettes. The cuvette is constructed from 1-cm i.d. square tubular glass with a 19/22 male drip-tip-type joint (C) attached to the top. The end of the drip-tip is cut off to accept a 7-mm rubber serum stopper (B) that separates the cuvette from the upper chamber. The female 19/22 joint is fitted with a 13-mm rubber serum stopper (A). An O_2 -scavenging solution can be placed in the upper chamber in the annular space above the ground-glass joint. The cuvettes are evacuated and flushed through a needle attached to a gassing manifold. Additions of enzyme or substrates are made by syringe through the serum stoppers. With the inner serum stopper removed, the entire gas phase is in contact with the O_2 -scavenging solution. The cuvettes are a modification of those described by Averill et al. (1978).

electrode. The H_2 electrode was calibrated by injecting aliquots of water equilibrated with 98 kPa (1 atm local conditions, 760 mmHg = 101.3 kPa) of H_2 into the electrode chamber. Each technique continuously monitored the reaction and could be used to generate progress curves (reaction allowed to proceed to completion) or initial velocity data. All reactions were carried out in 20 mM Mes buffer, pH 6.0, at 30 °C in the absence of O_2 (except for O_2 inhibition experiments). O_2 was removed from solutions by repeated evacuation and sparging with argon. Concentrations of gases in solution were calculated from their solubility coefficients (Lange, 1952) and partial pressures. Protein concentration was estimated by the dye-binding technique (Bradford, 1976).

In the CO inhibition experiments, the reaction progress was monitored spectrophotometrically (CO interferes with the H_2 electrode). The cuvettes were prepared and then preincubated for 2 h with an O_2 -scavenging solution (1 mM methyl viologen and 1 mM $Na_2S_2O_4$ in 50 mM Hepes, pH 7.5) in the upper chamber (the inner serum stopper was removed). In the CO vs. H_2 experiments, the reaction was initiated by addition of enzyme, and a small percentage of the total methylene blue then was reduced with the H_2 dissolved in the buffer; this generated a progress curve. Diffusion of H_2 from the gas phase to the buffer was sufficiently slow so the base line remained virtually flat. The cuvette then was shaken to reequilibrate the buffer with the gas phase, and an identical progress curve was obtained. The concentration of H_2 at various points along the progress curve was determined by calculating the difference in oxidized methylene blue concentration at each point and the base line for that progress curve. In the CO vs. methylene blue experiments, the procedure was similar, except that the methylene blue was allowed to become completely reduced. It was necessary to reequilibrate the solution with the gas phase several times by shaking the cuvette; rates were taken from the curve at points immediately after the reequilibration.

Reduced methylene blue is autooxidizable, so to use progress curves in the analysis of O_2 inhibition precautions must be

taken to hold the O_2 concentration steady during the reaction. This was accomplished by monitoring O_2 (with an O_2 electrode) and H_2 concentrations simultaneously and maintaining the O_2 concentrations by adding microliter quantities of O_2 -saturated buffer to the chamber as needed. The fluctuation in the O_2 concentration amounted to less than 10% of the indicated O_2 concentration. The enzyme was preincubated at each O_2 concentration for 1 min before H_2 was added to initiate the reaction. For determination of any enzyme inactivation during the course of the reaction, the O_2 was reduced by dithionite, and a new progress curve was generated and compared to a progress curve generated before O_2 was added.

In the D_2 - H_2O exchange experiments, traces of O_2 in the D_2 were removed as described for the CO inhibition experiments. The 17-mL reaction vials were shaken during the course of the reaction, and 1-mL gas samples were taken every 30 min. The gas samples were analyzed for H_2 , HD, and D_2 content with a Varian MAT 250 isotope ratio mass spectrometer.

Data Analysis. The nomenclature is that of Cleland (1963). The data were fit to various appropriate models with the computer programs of Cleland (1967). The K_m values and inhibition constants were calculated from the best fit of the data to the model. The lines in Figures 2–6 are generated from the fit while the points are actual data points.

Results

All experiments were carried out with highly purified hydrogenase preparations. The specific activity of a fresh preparation of enzyme ranged from 10 to 20 μmol of H_2 oxidized ($\text{min}\cdot\text{mg}$ of protein) $^{-1}$ with 100 μM methylene blue as the e^- acceptor. The protein is not homogeneous, but the activity corresponds to a single major band (50–70% of the total protein) on native gels. No major contaminants are apparent from the visible spectrum, and the EPR spectrum is typical of hydrogenases. Crude preparations of hydrogenase reduced O_2 in the presence of H_2 , but these purified preparations had lost this capability. While we cannot rule out the presence of contaminating electron mediators in our preparations, we have no evidence to indicate their presence. Hydrogenase remained active for several weeks when stored anaerobically at 4 °C, although the activity decayed slowly with a half-life of about 1 week. Preparations were used for as long as 3 weeks after purification.

Whenever possible, data were collected from progress curves, as this required little enzyme and produced data less variable than initial velocity data. Velocities were measured as tangents to the curve at selected concentrations of substrates. To test the validity of the progress curves, a second aliquot of the limiting substrate was added, and a second progress curve was produced. If the curves were identical, the data were accepted. Lags in activity up to 1 min frequently were observed, and they were at least partially attributable to traces of O_2 remaining in the reaction chamber. Data were not collected until the lags, if present, were overcome. With the amperometric technique, H_2 concentrations as low as 0.1 μM were measured routinely. With the spectrophotometric technique, e^- carrier concentrations were conveniently measured down to 1 μM .

Kinetics of H_2 Oxidation and e^- Carrier Reduction. Table I lists six e^- carriers and three kinetic constants associated with each e^- carrier. All reactions were carried out on the same day with the same hydrogenase preparation. The V_m values vary only by a factor of 2, with the exception of methyl viologen. The double-reciprocal plots were linear whether a one e^- or a two e^- carrier was used. The $K_m(H_2)$ values also vary only by a factor of 2, but the K_m values for the e^- carriers

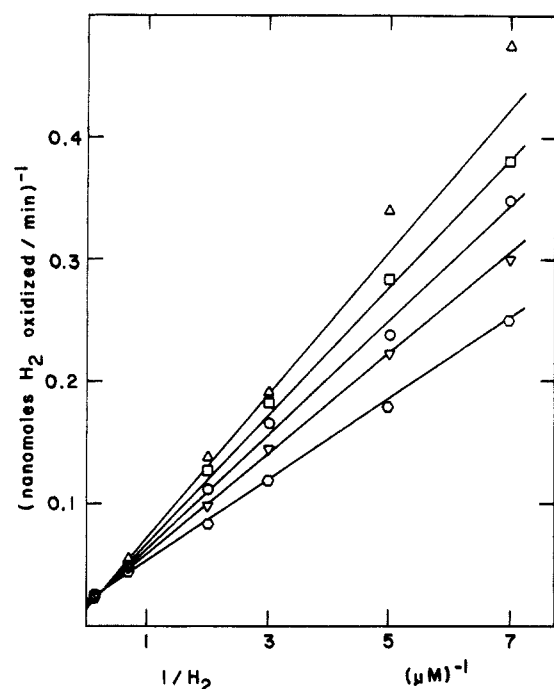


FIGURE 2: Double-reciprocal plot of v vs. H_2 at fixed inhibitory levels of methylene blue. Each reaction mixture contained $3.6 \mu\text{g}$ of protein. The data were collected from progress curves generated amperometrically. Although we interpreted the inhibition as competitive, the best computer fit of the data (indicated by the lines) was obtained when the intersection point was allowed to deviate from the ordinate. The lines intersect just to the right of the ordinate, and this indicates residual activation of hydrogenase by methylene blue at the high H_2 concentrations. The methylene blue concentrations were (\circ) 0.1, (∇) 0.4, (\square) 0.8, (\square) 1.2, and (Δ) 1.6 mM. Concentrations of 0.2 and 0.6 mM methylene blue were omitted from the figure for clarity.

range from $5.0 \mu\text{M}$ to 22 mM . Previous studies with bacteroids and purified enzyme have indicated that benzyl viologen and methyl viologen are poor acceptors, but saturation curves were not presented (Arp & Burris, 1979; Ruiz-Argüeso et al., 1979; Dixon, 1972). To test whether benzyl viologen or a contaminant was responsible for the activity, we twice recrystallized the benzyl viologen from methanol/acetone. Identical V_m and K_m values were obtained before and after purification. The K_m values we observed varied somewhat with the enzyme preparation. Values of the K_m for H_2 range from 0.97 to $2.6 \mu\text{M}$ (with methylene blue as the e^- carrier), and

Table I: Kinetic Constants^a Associated with Several Electron Acceptors

electron acceptor	$V_m^{b,c}$	$K_m(\text{acceptor})^c$ (mM)	$K_m(H_2)^d$ (μM)
ferricyanide	38 ± 3	0.0050 ± 0.0012	0.51 ± 0.21
dichlorophenol-indophenol	73 ± 14	0.053 ± 0.016	0.64 ± 0.17
phenazine methosulfate	68 ± 2	0.0066 ± 0.0012	1.02 ± 0.09
methylene blue	66 ± 3	0.017 ± 0.002	0.97 ± 0.07
benzyl viologen	30 ± 3	22 ± 4	0.55 ± 0.06
methyl viologen	~ 3	~ 16	0.86 ± 0.36

^a Values are computer fit to $v = VA/(K + A)$. ^b nmol of acceptor reduced (min- $5.2 \mu\text{g}$ of protein)⁻¹. ^c Determined spectrometrically under $750 \mu\text{M}$ H_2 . ^d Determined amperometrically at 100 mM methyl and benzyl viologen; all other acceptors at $100 \mu\text{M}$.

values of the K_m for methylene blue range from 6 to $17 \mu\text{M}$. Initial velocity patterns were determined with methylene blue as the electron acceptor; the pattern intersects to the left of the ordinate [data not shown; see Arp (1980)].

Inhibition Studies of H_2 Oxidation: Product Inhibition. No significant inhibition of V_m or V/K for methylene blue, phenazine methosulfate, or H_2 was observed by concentrations of the reduced e^- carrier up to $100 \mu\text{M}$. Experiments at higher concentrations were complicated by precipitation of the reduced dyes. Protons are a product of H_2 oxidation, and whereas the hydrogenase activity is pH dependent, no attempt was made to separate product effects from other ionization effects. We did, however, examine the effect of Li^+ and Na^+ as potential proton analogues and observed no inhibition at concentrations up to 10 mM .

Substrate Inhibition. High concentrations of oxidized methylene blue inhibit H_2 oxidation, particularly at low H_2 concentrations. The inhibition is reversible as determined by preincubating the enzyme in 1 mM methylene blue, and then diluting the methylene blue to $100 \mu\text{M}$ prior to assaying. The reaction velocity was determined as a function of H_2 concentration at fixed inhibitory levels of methylene blue (Figure 2). The inhibition is competitive vs. H_2 with a K_I of $2.4 \pm 0.6 \text{ mM}$. Phenazine methosulfate also exhibits substrate inhibition ($K_I \sim 100 \mu\text{M}$). No substrate inhibition of H_2 oxidation by H_2 was observed at concentrations of H_2 up to $750 \mu\text{M}$.

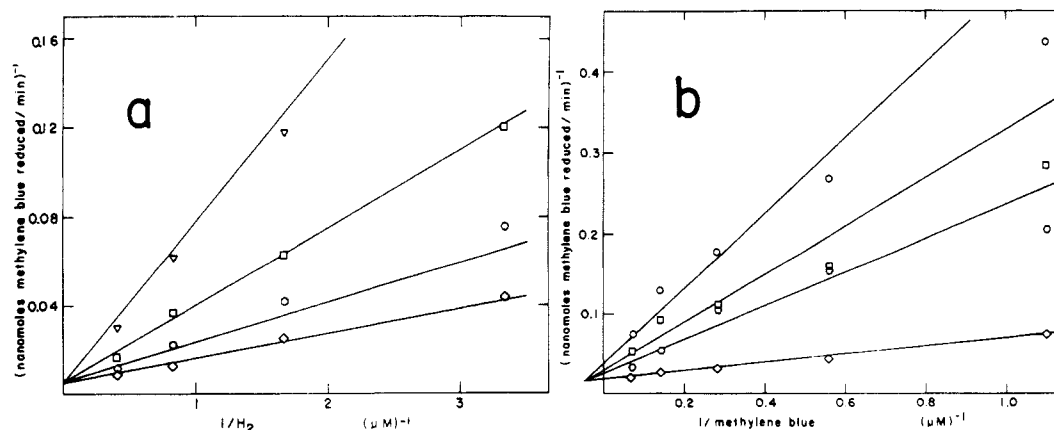


FIGURE 3: (a) CO inhibition of hydrogenase with H_2 plotted as substrate and methylene blue as acceptor. Each reaction mixture contained $1.0 \mu\text{g}$ of protein. Data were collected from progress curves as described under Materials and Methods. The lines indicate the computer fit of the data to competitive inhibition. The CO concentrations were (\diamond) 0, (\circ) 81, (\square) 301, and (∇) 810 μM . Each plotted point is an average of at least three data points. The methylene blue concentration was initially $100 \mu\text{M}$. (b) CO inhibition of methylene blue reduction by H_2 with methylene blue plotted as substrate. Each reaction mixture contained $1.0 \mu\text{g}$ of protein. Each point plotted is the average of two data points. The lines indicate the computer fit to noncompetitive inhibition. See Materials and Methods for details. The CO concentrations were (\diamond) 0, (\circ) 253, (\square) 371, and (∇) 514 μM . The H_2 concentration was $0.5 \mu\text{M}$.

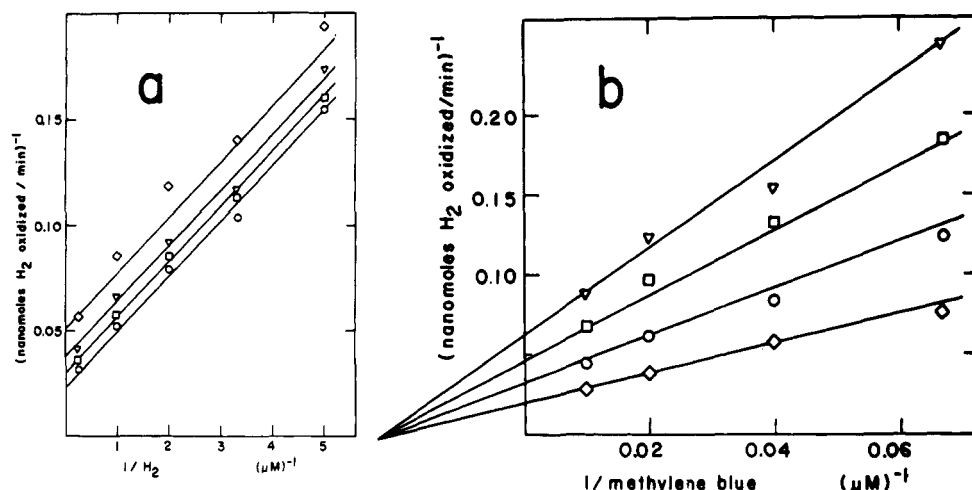


FIGURE 4: (a) O_2 inhibition of hydrogenase with H_2 plotted as substrate. Each reaction mixture contained $10.4 \mu\text{g}$ of protein. The data were collected from progress curves generated amperometrically as described under Materials and Methods. The lines indicate the computer fit of the data to uncompetitive inhibition. The O_2 concentrations were (\circ) 0, (\square) 3.6, (∇) 7.2, and (\diamond) $12.8 \mu\text{M}$. The methylene blue concentration was $100 \mu\text{M}$. (b) O_2 inhibition of hydrogenase with methylene blue plotted as substrate. Each reaction mixture contained $3.6 \mu\text{g}$ of protein. See Materials and Methods for details. The lines indicate the computer fit of the data to noncompetitive inhibition. The O_2 concentrations were (\diamond) 0, (\circ) 6.3, (\square) 12.6, and (∇) $20 \mu\text{M}$. The H_2 concentration was $50 \mu\text{M}$.

CO Inhibition. No inhibition by CO (up to $810 \mu\text{M}$) was observed if the H_2 concentration was saturating; however, when H_2 was limiting, then CO inhibition became apparent. The inhibition is competitive vs. H_2 (Figure 3a) with a K_I of $157 \pm 17 \mu\text{M}$ CO. To observe any inhibition with methylene blue as the variable substrate, it was necessary to fix the H_2 concentration below its K_m . The data in Figure 3b were fit to noncompetitive inhibition, and a $K_{I,\text{slope}}$ of $92 \pm 24 \mu\text{M}$ CO and $K_{I,\text{intercept}}$ of $700 \pm 420 \mu\text{M}$ CO were observed. When the CO was removed by evacuation, the inhibition was reversed.

O_2 Inhibition. O_2 inactivates purified rhizobial hydrogenases, and the half-life of the enzyme is about 1 h (Arp & Burris, 1979). However, O_2 also can act as an inhibitor of H_2 oxidation, and this inhibition is completely reversible if the exposure to O_2 is at low concentrations for short times. We examined the pattern of O_2 inhibition with either H_2 or methylene blue as the variable substrate. The inhibition vs. H_2 apparently is uncompetitive (Figure 4a) with $K_{I,\text{intercept}} = 10.7 \pm 1.0 \mu\text{M}$ O_2 . At higher O_2 concentrations than those shown, some inactivation of the hydrogenase occurred during the reaction. The inhibition is noncompetitive (Figure 4b) vs. methylene blue ($K_{I,\text{slope}} = 10.7 \pm 2.1 \mu\text{M}$; $K_{I,\text{intercept}} = 9.5 \pm 1.4 \mu\text{M}$). The inhibition by O_2 is not immediate but requires about 1 min to reach a maximum level. In experiments with no e^- acceptor other than O_2 , there was no H_2 oxidation. Thus, O_2 will not accept electrons from the purified hydrogenase.

C_2H_2 Inhibition. Several investigators have reported that C_2H_2 inhibits H_2 oxidation in certain organisms (Smith et al., 1976; Bothe et al., 1977; Van der Werf & Yates, 1978). Therefore, we examined the effect of C_2H_2 on purified rhizobial hydrogenase activity. When the reaction was initiated by addition of enzyme, we observed no inhibitory effect on V_m or V/K for H_2 or methylene blue up to 98 kPa of C_2H_2 . When the enzyme was preincubated in the presence of 98 kPa of C_2H_2 and the absence of H_2 prior to assaying, the reaction rates were decreased relative to controls preincubated under argon. The magnitude of the decrease in activity was dependent upon the length of incubation (47% decrease in 50 min, 95% in 100 min), and the decrease was not reversible; i.e., inactivation occurred. Precautions were taken to remove traces of O_2 from the C_2H_2 prior to the preincubation.

H_2 Evolution Studies. We previously have demonstrated H_2 evolution by rhizobial hydrogenase with reduced methyl

viologen as the electron donor (Arp & Burris, 1979). Maximal rates of H_2 evolution at pH 6.0 are 1–3% of the maximal rates of H_2 oxidation. Dithionite alone will not support H_2 evolution, nor will reduced methylene blue. We have used dithionite-reduced benzyl viologen and methyl viologen to support H_2 evolution. The K_m for methyl viologen is $11.0 \pm 1.0 \mu\text{M}$.

The time course of H_2 evolution was nonlinear because of product inhibition by H_2 as follows: (1) When the rate of H_2 evolution approached zero, additional portions of enzyme did not stimulate further activity. (2) When H_2 was added to the reaction chamber prior to enzyme addition, no H_2 was evolved. (3) When accumulated H_2 was removed from the assay chamber by sparging, H_2 evolution began again and generated a curve identical with that generated before H_2 was added. Evolution does not decrease due to an unfavorable equilibrium because dithionite is present in excess and will keep methyl viologen reduced. The pattern of H_2 inhibition indicated noncompetitive inhibition vs. methyl viologen with a $K_{I,\text{slope}} = 0.48 \pm 0.07 \mu\text{M}$ H_2 and a $K_{I,\text{intercept}} = 0.42 \pm 0.06 \mu\text{M}$ H_2 (Figure 5). Even with 1 mM methyl viologen, H_2 still is a potent inhibitor of H_2 evolution.

D_2 - H_2O Exchange Reaction. Hydrogenase was incubated in the presence of D_2 for a period, and the gas phase was examined for mass 2 (H_2), mass 3 (HD), and mass 4 (D_2). Mass 2 or 3 increases from an exchange of deuterons (formed when D_2 is split) with protons in the buffer, re-formation of the dihydrogen bond, and release of either H_2 or HD. We observed both H_2 and HD formation catalyzed by hydrogenase (Figure 6) although the rates were low. The time course of H_2 and HD formation was linear for at least 5 h. Heat-inactivated enzyme did not catalyze the exchange. The rates of H_2 and HD formation were the same when the assay solution contained 0.1 mM dithionite and when the dithionite had been oxidized by titration with methylene blue. The exchange rates depicted in Figure 6 correspond to 36 nmol of H_2 formed/min and 6 nmol of HD formed/min. This compares to a H_2 evolution rate of 9 nmol/min when supported by 1 mM reduced methyl viologen and a H_2 oxidation rate of 665 nmol/min when supported by $100 \mu\text{M}$ methylene blue where the same amount of protein was used for each reaction.

Discussion

The results are consistent with the kinetic scheme presented

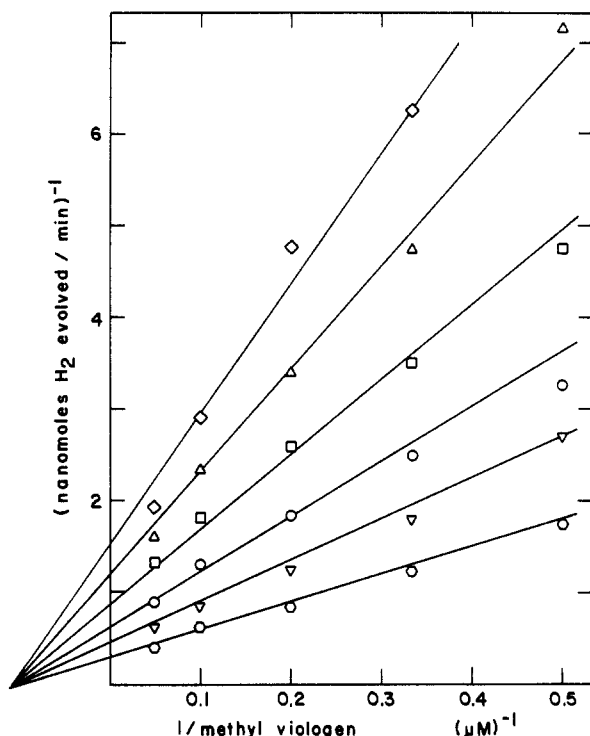


FIGURE 5: H_2 inhibition of H_2 evolution. Each reaction mixture contained $11.5 \mu\text{g}$ of protein. Velocities were taken at the following H_2 concentrations from inhibition curves generated at various concentrations of reduced methyl viologen: (\circ) 0, (∇) 0.24, (\circ) 0.48, (\square) 0.83, (Δ) 1.31, and (\diamond) 1.78 μM . The lines indicate the computer fit of the data to noncompetitive inhibition. Dithionite (1 mM) was present in all reaction mixtures.

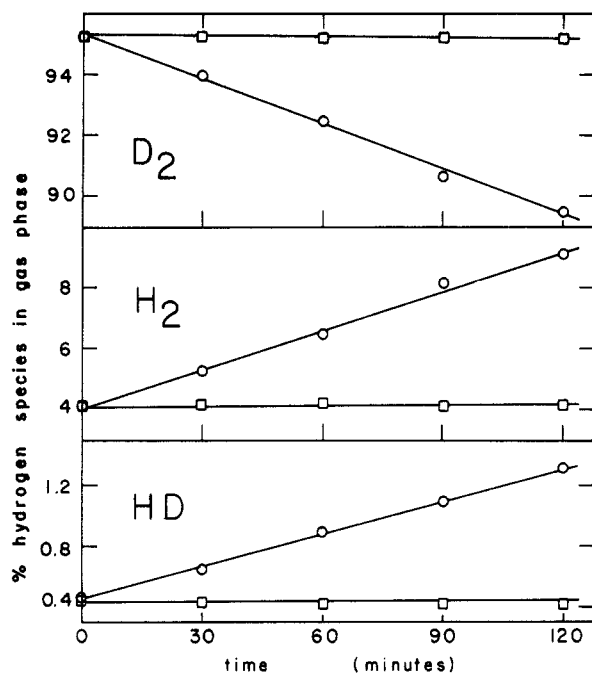


FIGURE 6: D_2 - H_2O exchange reaction. Each reaction mixture contained $46 \mu\text{g}$ of protein in 1 mL of buffer (+0.1 mM dithionite). (\circ) Sample; (\square) heat-inactivated control. The gas phase consisted of 10% D_2 and 90% argon at a total pressure of 98 kPa (1 atm).

in Figure 7. We propose a two-site ping-pong mechanism in which the enzyme intermediate (E') is the reduced enzyme and the two sites serve for the binding of H_2 and the e^- carrier. Intersecting initial velocity patterns indicate a reversible pathway between the binding of the two substrates. Normally this suggests a sequential mechanism, but in this case does not exclude a ping-pong mechanism, because protons (a reaction

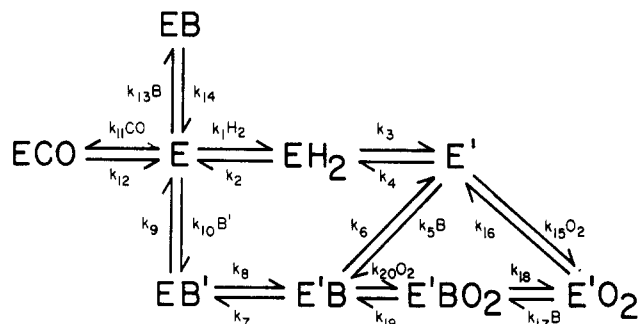


FIGURE 7: Model for the kinetic mechanism of rhizobial hydrogenase. E, oxidized enzyme; E' , reduced enzyme; B, oxidized e^- carrier; B' , reduced e^- carrier. k_1 , k_2 , etc. are rate constants.

product) are always present and provide a reversible pathway between the binding of both substrates. [For a detailed discussion of initial velocity patterns, see Cleland (1970).] The existence of the D_2 - H_2O exchange reaction shows that the formation of E' can be reversed in the absence of the e^- carrier. In this reaction, both H_2 and HD are formed. The exchange reaction is consistent with a ping-pong mechanism and inconsistent with a sequential mechanism, since binding of the e^- carrier need not occur before release of the protons. However, with the specified conditions, the total flux of electrons in the exchange reaction (formation of $H_2 + HD$) is only 6.5% of the flux of electrons involved in H_2 oxidation. The simplest explanation for the low electron flux to form $H_2 + HD$ is that exchange is limited by one or more of the reverse reactions, i.e., reassociation and reduction of the protons (k_4) and release of H_2 (k_2). This explanation also is consistent with the low rate observed for H_2 evolution from reduced methyl viologen. However, we cannot exclude more complicated mechanisms that are compatible with the two-site mechanism (e.g., during H_2 -dependent e^- carrier reduction by hydrogenase, protons could be released after binding, reduction, and/or release of the e^- carrier), and, hence, we have not included protons in the reaction scheme.

H_2 is a powerful inhibitor of H_2 evolution (Figure 5), and although H_2 and the reduced e^- carrier both compete for the oxidized enzyme, the inhibition is not competitive (competitive inhibition would be expected if both were binding to the same site). Because the inhibition is clearly noncompetitive, the reduced e^- carrier and H_2 must be binding to different sites on E. O_2 is an uncompetitive inhibitor vs. H_2 , so it binds after H_2 , and we have indicated O_2 binding to E' . The inhibition by O_2 is noncompetitive vs. methylene blue, and this requires two sites on E' also, although not necessarily the same two sites as for H_2 and the reduced e^- carrier on E. It is reasonable to assume that e^- carriers, whether reduced or oxidized, will bind to the second site. The CO and substrate inhibition data also can be interpreted in terms of a two-site mechanism. CO is a competitive inhibitor of H_2 and noncompetitive vs. methylene blue (Figure 3a,b); thus, CO and H_2 apparently compete for the same site on the oxidized enzyme while methylene blue binds at the other site. The substrate inhibition by oxidized methylene blue (Figure 2) is competitive vs. H_2 , indicating that the binding is mutually exclusive. While this could indicate that H_2 and methylene blue bind to the same site, it seems more likely (given their chemical structures) that the binding of oxidized methylene blue to E prevents the binding of H_2 at the other site.

With methylene blue and phenazine methosulfate as e^- carriers, the reaction is irreversible (no H_2 evolution), and there is no product inhibition by reduced methylene blue or phenazine methosulfate. As all of the H_2 oxidation experiments

were performed with these e^- carriers, it would be interesting to repeat some of the experiments with the viologens, which are reversible e^- carriers in this system. For the accommodation of one e^- carrier, three additional steps must be added to the scheme to include binding, reduction (or oxidation), and release of the second e^- carrier needed to complete the reaction. Because double-reciprocal plots were linear for one e^- carrier, the second molecule must bind in a ping-pong fashion.

Multisite ping-pong kinetics are observed in several biotin (Northrop, 1969; Barden et al., 1972) and lipoic acid (Tsai et al., 1973) containing enzymes that have a flexible arm to reach the different sites. Glutamate synthase also exhibits multisite ping-pong kinetics (Rendina & Orme-Johnson, 1978); electrons are presumed to flow from one site to the other via the iron-sulfur and flavin prosthetic groups. Such a model might be applicable to hydrogenases, as they also contain iron-sulfur prosthetic groups (Schlegel & Schneider, 1978). H_2 could be reversibly activated at one site, and the iron-sulfur center(s) could serve to store electrons and to transfer electrons reversibly from the H_2 site to the e^- carrier site.

CO inhibition of hydrogenase activity has been observed with several organisms (Mortenson & Chen, 1974) but not in soybean nodule bacteroids (Ruiz-Argüeso et al., 1979). However, the partial pressure of H_2 in the bacteroid experiments was 0.1 atm (80 μ M), sufficient to saturate the system and prevent any CO inhibition. For rhizobial hydrogenase, the inhibition constant for CO (157 μ M) is over 25 times the value of 5.8 μ M reported for *Clostridium pasteurianum* hydrogenase (Erbes & Burris, 1978). It is interesting that the K_m values for H_2 follow the reverse pattern; the K_m for H_2 for the clostridial hydrogenase is 386 μ M H_2 (at pH 8.0), whereas the K_m for H_2 for the rhizobial hydrogenase is near 1 μ M.

C_2H_2 has been shown to be an inhibitor of hydrogenase activity in some organisms (Smith et al., 1976; Bothe et al., 1977; Van der Werf & Yates, 1978), but the inhibition does not appear to be general for hydrogenases (Houchins, 1980; Emerich et al., 1979), and no inhibition of H_2 oxidation by bacteroids has been observed (Ruiz-Argüeso et al., 1979). We saw no immediate inhibition by up to 98 kPa (1 atm) of C_2H_2 . With the solubilized enzyme from *Azotobacter chroococcum*, a preincubation under C_2H_2 in the absence of H_2 was necessary to produce inhibition (Van der Werf & Yates, 1978). This procedure with the rhizobial hydrogenase gave us irreversible inactivation of the enzyme.

Exposure to O_2 inactivates the hydrogenases from several organisms, whereas other hydrogenases appear completely stable to O_2 . However, a clear distinction between inactivation and reversible inhibition of hydrogenases is not always made. The rhizobial hydrogenase is inactivated in air with a half-life of about 1 h, but exposure to low levels of O_2 for short times gives a completely reversible inhibition of hydrogenase activity. O_2 , the terminal e^- acceptor in the H_2 oxidation reaction in vivo, will not accept electrons directly from hydrogenase. In whole bacteroids, hydrogenase is very stable toward O_2 and does not appear to be inhibited by O_2 (Ruiz-Argüeso et al., 1979).

H_2 -oxidizing hydrogenases generally have very low rates of H_2 evolution in the presence of electron donors (Arp & Burris, 1979; Van der Werf & Yates, 1978; Chen & Blanchard, 1978; Houchins, 1980), and this is true of the rhizobial hydrogenase. The inhibition of H_2 evolution by H_2 has not been reported previously for this type of hydrogenase. H_2 evolution by clostridial hydrogenase was reduced only 8% when the buffer was saturated with H_2 (Erbes & Burris, 1978). D_2 - H_2O exchange rates also are very low for H_2 -oxidizing hydrogenases

relative to other hydrogenases (Krasna & Rittenberg, 1956; Hyndman et al., 1953; Dixon, 1972). Exchange experiments have been useful in elucidating the mechanism of hydrogenase (Krasna & Rittenberg, 1954; Tamiya & Miller, 1963; Yagi et al., 1973). A comparative study of the exchange properties of H_2 -oxidizing hydrogenases and bidirectional hydrogenases should be informative.

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Evidence for an Intrazymogen Mechanism in the Conversion of Proacrosin into Acrosin[†]

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ABSTRACT: The mechanism responsible for the spontaneous initiation of proacrosin conversion into acrosin in vitro was studied and characterized by using the highly effective inhibitor leupeptin. In the presence of excess leupeptin [(10⁻²–10⁻³)K_i to acrosin], proacrosin spontaneously and completely converted into acrosin at pH 8. However, only the initial enzyme product, m_α-acrosin, was produced, and the rate of conversion was not affected when exogenous m_α-acrosin was added to the reaction mixture. These results demonstrate that excess leu-

peptin eliminated all conversion and degradative reactions requiring active acrosin. Kinetically, the conversion of proacrosin into m_α-acrosin in the presence of excess leupeptin appeared first order. The observed half-life (*t*_{1/2} = 1.4 h) did not vary over a 10-fold range of leupeptin or initial proacrosin concentrations. These data demonstrate that proacrosin can self-catalyze its own conversion into m_α-acrosin by an intrazymogen mechanism.

Acrosin is a serine proteinase of trypsin-like substrate specificity associated with the sperm cell acrosome. The majority of potential acrosin present in mammalian epididymal or ejaculated spermatozoa, however, is a zymogen form, proacrosin (Meizel, 1972; Meizel & Mukerji, 1975; Tobias & Schumacher, 1977; Polakoski & Parrish, 1977; Harrison & Brown, 1979). Since acrosin activity is essential for fertility [reviewed by Zaneveld et al. (1975)], it is therefore important to understand the mechanisms and regulation of the conversion of proacrosin into its catalytically active enzyme form(s).

In vitro, purified proacrosin spontaneously and rapidly converts into acrosin at neutral pH. The reaction occurs by limited proteolysis of the zymogen and appears "auto-catalyzed", i.e., accelerated by exogenously added and/or endogenously generated acrosin (Meizel & Mukerji, 1975; Polakoski & Parrish, 1977). A minimum mechanism describing the conversion of proacrosin can thus be written



where Z and E are the zymogen and enzyme, respectively.

Equation 1, however, cannot satisfactorily explain how the first molecules of acrosin evolve, especially from highly purified preparations of proacrosin. Although it is possible that the reaction may be initiated by other proteinases, this explanation is unlikely for purified proacrosin as has been considered in detail elsewhere (Polakoski & Parrish, 1977; Parrish et al., 1978) and further shown in this report. Rather, alternative mechanisms for the initiation of proacrosin conversion into acrosin need to be considered.

Besides catalysis by the generated enzyme or some contaminating proteinase, only two other limited proteolytic mechanisms for zymogen conversion into enzyme are currently thought possible: inter- and intrazymogen-catalyzed con-

versions [reviewed by Kassel & Kay (1974) and Neurath & Walsh (1976)]. Trypsinogen can convert into trypsin via an interzymogen mechanism (Kay & Kassel, 1971) as is represented by



Alternatively, pepsinogen can convert into pepsin via an intrazymogen mechanism (Bustin & Conway-Jacobs, 1971)



These latter mechanisms involving zymogen self-catalyzed conversions (eq 2 and 3) may be kinetically differentiated from the enzyme-catalyzed reaction (eq 1). However, McPhie (1972) has cautioned that direct kinetic analyses of the zymogen conversion profiles are highly subject to misinterpretation if the enzyme-catalyzed mechanism (eq 1) predominates even at low initial zymogen concentrations and if degradation of the enzyme products occurs simultaneous with zymogen activation. Both of these events are known to occur in the conversion of proacrosin into acrosin (Polakoski & Parrish, 1977; Parrish & Polakoski, 1978). These difficulties may be circumvented by the use of an inhibitor which suppresses the enzyme-catalyzed conversion or degradation reactions but does not prevent the conversion mechanism catalyzed by the zymogen (eq 2 or 3). In this work, leupeptin, a highly effective acrosin inhibitor, was found to satisfy this latter requirement. Kinetic analyses of the spontaneous conversion of proacrosin into acrosin in the presence of excess leupeptin demonstrated that the reaction can occur by the intrazymogen mechanism shown in eq 3.

Experimental Procedures

Materials. BzArgOEt,¹ BzArgpNA, and leupeptin were purchased from the Sigma Chemical Co. and used without

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¹ Abbreviations used: leupeptin, *N*-acetyl-L-leucyl-L-leucyl-L-argininal; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester hydrochloride; BzArgpNA, *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; Tris, tris(hydroxymethyl)aminomethane; NaDodSO₄, sodium dodecyl sulfate; *M*_r, relative molecular mass; LBTI, lima bean trypsin inhibitor.