Oxygen Effects on the Nickel- and Iron-Containing Hydrogenase from *Azotobacter vinelandii*†

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ABSTRACT: The effects of O_2 on membrane-bound *Azotobacter vinelandii* hydrogenase in both the membrane-associated and purified states were characterized. O_2 was a rapid-equilibrium, reversible inhibitor both of H_2 oxidation/electron acceptor reduction activity and of the exchange activity. O_2 inhibition was noncompetitive versus the electron acceptor methylene blue and uncompetitive versus the substrate H_2. The inhibition constant (K_i) for purified enzyme was 5.5 ± 0.6 μM and for membrane-associated enzyme was 2.5 ± 0.5 μM. O_2 inhibition was rapidly reversed by removal of the O_2. In addition to an O_2-dependent, reversible inhibition of hydrogenase, a time-dependent, irreversible inactivation by O_2 of both H_2 oxidation and exchange activities for this hydrogenase was demonstrated. The irreversible inactivation followed a first-order process, with a half-life ($t_{1/2}$) for purified enzyme of 5.9 ± 0.9 min and a half-life for membrane-associated enzyme of 46.1 ± 2.0 min. The inactivation by O_2 was protected against by the substrate H_2 in a concentration-dependent manner. This is the first report of H_2 protection from O_2-dependent, irreversible inactivation for any hydrogenase. The competitive inhibitor CO did not affect the irreversible inactivation by O_2 or the protection by H_2. A model is proposed that outlines various oxidation states of this hydrogenase and the effects of O_2 on each state.

Hydrogenases (EC classes 1.12 and 1.18) are metalloenzymes found in a diverse group of bacteria, several algae, and a few fungi which catalyze the consumption or evolution of the simplest molecule dihydrogen. While hydrogenases are a rather heterogeneous group of enzymes, there are a few properties that these enzymes have in common (Adams et al., 1981). First, all hydrogenases contain non-heme iron in the form of iron–sulfur centers. The largest subgroup of hydrogenases also contains nickel (Cammack et al., 1988). The exact catalytic role of these metal centers in hydrogenases is unknown. A second characteristic that hydrogenases share is interaction in some way with oxygen [see Cammack et al. (1988) for a review]. Despite this commonality of interaction with oxygen, the nature of this interaction appears to be quite variable among hydrogenases, and there is no general consensus in the literature on these effects.

The effects of oxygen on hydrogenases seem to fall into three categories. First, a large number of hydrogenases, principally the Ni- and Fe-containing dimers, can be purified in the presence of O_2 in an inactive state and can be activated by removal of oxygen followed by reduction with H_2 or other reductant (i.e., dithionite). The time course of activation varies widely between hydrogenases, ranging from seconds to hours (Cammack et al., 1988). This group of hydrogenases, therefore, can exist in a reversibly inactive state that is stable toward O_2. Second, nearly all hydrogenases are susceptible to irreversible inactivation by exposure to O_2 when in the active state regardless of the history of the enzyme. The half-times for these inactivations vary between 2 min and several hours (Cammack et al., 1988). A notable exception to this irreversible inactivation appears to be the Ni- and Fe-containing hydrogenase purified from *Desulfovibrio gigas* which is reversibly inactivated by exposure to O_2 and can be slowly (4 h) reactivated by H_2 (Fernandez et al., 1985). Finally, it has been reported for a few hydrogenases that O_2 can inhibit the active state. Inhibition differs from inactivation in that removal of O_2 from inhibited enzyme results in full recovery of activity, while simply removing O_2 does not return inactive enzyme to an active state. Inhibition by O_2 has been characterized for the membrane-bound hydrogenases purified from the aerobic microorganisms *Bradyrhizobium japonicum* (Arp & Burris, 1981) and *Alcaligenes eutrophus* (H16) (Schink & Probst, 1980).

In the present work, we have investigated the effects of O_2 on the membrane-bound hydrogenase from the nitrogen-fixing microorganism *Azotobacter vinelandii*. We demonstrate that O_2 acts both as a rapid-equilibrium, reversible inhibitor and as a slower, irreversible inactivator of both the membrane-associated and purified forms of this hydrogenase. We propose a model that summarizes the various states of this hydrogenase and the effects of O_2 on each of these states.

Experimental Procedures

Materials. Hydrogen, nitrogen, oxygen, and argon (>99.99% purity) were purchased from Liquid Carbonic Corp. (Chicago, IL). Hydrogen, nitrogen, and argon were passed over a heated, copper-based catalyst (R3-11; Chemical Dynamics Corp., South Plainfield, NJ) to remove residual oxygen. Carbon monoxide (99.99% purity) was purchased from Matheson Gas Products (Cucamonga, CA) and was used without further purification. Carrier-free $^3H_2$ gas was obtained from Lawrence Berkeley Laboratory (Berkeley, CA). Liquid scintillation fluid was purchased from National Diagnostics, Inc. (Somerville, NJ). The detergent Emulgen 913 was from Karlan Chemical Corp. (Torrance, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Growth of Azotobacter vinelandii Cells and Preparation of Membranes. A. vinelandii cells (strain OP) were cultured in a 50-L fermentor (New Brunswick Scientific, Edison, NJ) under nitrogen-fixing conditions (Strandberg & Wilson, 1968) with aeration (20 L·min^{-1}). H_2 oxidation activity coupled to methylene blue reduction was monitored amperometrically

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(Sweet et al., 1980) in 10 mM phosphate buffer, pH 7.0, including 100 μM methylene blue, 90 mM NaF, and 2 mM EDTA at 24 °C (Partridge et al., 1980). Cells were harvested at approximately 2 ODU (600 nm) by tangential flow ultrafiltration (Millipore Corp., Bedford, MA) when the hydrogenase specific activity peaked at 300 nmol of H_2 oxidized·min^{-1}·(mg of total cell protein)$^{-1}$. The cells were lysed by osmotic shock as described (Hageman et al., 1980) followed by low-speed centrifugation (5000*g*, 5 min) to remove unbroken cells. The membranes were then collected by centrifugation at 100000*g* for 1 h followed by resuspension in 20 mM Tris buffer, pH 7.4, containing 0.5 M NaCl. The washed membranes were collected by centrifugation at 100000*g* for 1 h. The membranes were further washed by resuspension in 20 mM Tris buffer, pH 7.4, containing 1 mM EDTA followed by centrifugation at 100000*g* for 1 h. The washed membranes had a specific activity of 2.5 μmol of H_2 oxidized·min^{-1}·(mg of total protein)$^{-1}$ in a H_2 oxidation/methylene blue reduction assay and were no longer capable of H_2 oxidation linked to O_2 reduction. These washed membranes were stored at −70 °C and were subsequently used for all experiments utilizing membranes or as a source for purification of hydrogenase.

Purification of A. vinelandii Hydrogenase from Membranes. The purification and subunit characterization of *A. vinelandii* hydrogenase have been reported (Seefeldt & Arp, 1986). The following section describes a modification of that purification procedure. Seventy-five grams of washed membranes (above) was resuspended in 300 mL of 20 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 0.4% (v/v) of the nonionic detergent Emulgen 913. For purification under anaerobic conditions, all buffers were evacuated and flushed several times with N_2 or Ar and included 2 mM dithionite ($Na_2S_2O_4$). The resuspended membranes were solubilized for 1 h at 30 °C followed by centrifugation at 100000*g* for 1 h. The resulting supernatant (detergent extract of membranes) was then dialyzed against 12 L of 10 mM succinate buffer, pH 5.0, for 14 h under argon. The dialyzed sample was then applied to a carboxymethyl-Sepharose column that had previously been equilibrated with 10 mM succinate buffer, pH 5.0. The column, with the hydrogenase bound to the top, was then washed with 3 column volumes of 10 mM succinate buffer, pH 5.0. Detergent was not included in the dialysis buffer or in any column buffers. The hydrogenase band was then selectively eluted with 10 mM succinate buffer, pH 5.0, containing 150 mM NaCl. The hydrogenase was loaded directly onto an octyl-Sepharose column that had been previously equilibrated with 20 mM Tris buffer, pH 7.4. The hydrogenase bound to the top of the column as a brown band. The column was washed with 3 column volumes of 20 mM Tris buffer, pH 7.4. Hydrogenase was eluted from the column with 20 mM Tris buffer, pH 7.4, containing 40% (v/v) ethanol. The preparation yielded 8–9 mg of hydrogenase protein that had a specific activity of 135 units·(mg of protein)$^{-1}$ as determined spectrophotometrically with methylene blue as the electron acceptor (pH 6.0, 30 °C). One unit of activity is defined as 1 μmol of H_2 oxidized·min^{-1}. Analysis of the purified enzyme by SDS–polyacrylamide electrophoresis revealed that the enzyme was nearly homogeneous (>95% pure).

Protein was determined by the method of Biuret (Gornall et al., 1949) with bovine serum albumin as standard.

A. vinelandii hydrogenase was also purified under aerobic conditions without the addition of dithionite (Seefeldt & Arp, 1987). In this state, the enzyme required deoxygenation followed by incubation with dithionite or H_2 for activation. It should be noted that the yield of this aerobically purified enzyme was routinely much lower than for enzyme prepared anaerobically (2% of anaerobically prepared). The specific activity was also lower, averaging about 75 units·(mg of protein)$^{-1}$ compared to 135 units·(mg of protein)$^{-1}$ for anaerobically purified enzyme.

H_2 Oxidation Assays. H_2 oxidation/electron acceptor reduction activity was determined spectrophotometrically at 30 °C in 50 mM MES buffer, pH 6.0, including either 150 μM methylene blue or 100 mM benzylviologen. All assays were performed in stoppered glass cuvettes with solutions made anaerobic by purging with H_2 gas. The following electron acceptors and extinction coefficients (ϵ) were used: methylene blue, ϵ = 11.4 mM^{-1}·cm^{-1} at 690 nm; benzyl viologen, ϵ = 8.3 mM^{-1}·cm^{-1} at 600 nm.

Reversible Inhibition by O_2. To determine the rate and extent of O_2 consumption by reduced electron carriers under assay conditions, the following control experiments were performed. Cuvettes were completely filled with buffer containing electron carrier and were made anaerobic by bubbling with H_2. Sufficient dithionite (from a 100 mM stock solution in buffer) was added to the anaerobic cuvette to reduce half of the oxidized electron carrier. Water saturated with O_2 (1.2 mM in solution) was injected into the cuvette, and the time course of the reduction of O_2 coupled to the reoxidation of reduced electron carrier was monitored spectrophotometrically.

For O_2 inhibition studies, anaerobically purified hydrogenase or deoxygenated membranes were injected into a H_2-saturated, H_2 oxidation/methylene blue reduction assay cuvette (described above), and the progress of the reaction was monitored spectrophotometrically. At a set elapsed time into the assay, the cuvette was removed from the spectrophotometer, and a volume of O_2-saturated water was injected into the cuvette. The cuvette was immediately replaced into the spectrophotometer, and the progress of H_2 oxidation/methylene blue reduction was monitored for an additional 20 s. This was repeated with a new cuvette and a different volume of O_2-saturated water added. The methylene blue reduction rate after the addition of O_2 was determined, and the concentration of O_2 in solution in the cuvette was calculated from the Bunsen coefficient (α = 0.0283 at 25 °C) (Dean, 1985). These experiments were repeated under conditions of saturating H_2 (688 μM in solution) and subsaturating methylene blue (17 μM) or under conditions of saturating methylene blue (132 μM) and subsaturating H_2 (1 μM). From plots of the reciprocal of the inhibited velocity ($1/v$) versus O_2 concentration in solution (Dixon plots), the K_i for O_2 and the type of inhibition versus H_2 and methylene blue were determined.

The reversibility of the O_2 inhibition was demonstrated by addition of deoxygenated leghemoglobin to the assay cuvette following the addition of O_2. Leghemoglobin was isolated and partially purified from soybean nodules essentially as described (Bergerson & Turner, 1979) and was a gift from Madeline Rasche.

Incubation Conditions. Incubations utilized stoppered serum vials (13.8 mL) that contained an inner, open-topped reaction chamber (1 mL) as previously described (Hyman & Arp, 1987). The indicated gas compositions were prepared by addition of the various gases to a N_2-purged vial. The final gas pressure was 101 kPa at 25 °C. N_2-purged buffer (20 mM HEPES, pH 7.5, unless stated otherwise) was added to the inner chamber after the indicated gas phase had been prepared, and the vials were allowed to equilibrate for 15 min with shaking prior to addition of enzyme. Purified hydrogenase or washed membranes were added to the inner vial to initiate

the incubation, which proceeded with shaking. Control experiments with buffer containing 2 mM dithionite confirmed that all of the dithionite carried with the enzyme was consumed by the O_2 within 15 s after addition of the sample to the incubation vial. In addition, all experiments were repeated with faster shaking rates and in larger vials and confirmed that gas diffusion was not limiting. At the indicated times, an aliquot was removed from the vial and either injected into an assay cuvette or placed into a separate, stoppered vial and repeatedly evacuated and flushed with H_2 followed by the addition of dithionite to 2 mM. These latter samples were allowed to incubate for a minimum of 1 h under these conditions before assay. Membrane-associated hydrogenase was assayed with methylene blue as the electron acceptor. Purified hydrogenase was assayed with benzyl viologen or methylene blue as the electron acceptor as noted. Reported H_2 oxidation rates were the maximum rates achieved during the course of the assay.

The percent activity remaining versus time was fit by computer iterations to a monophasic, exponential model $y = S + Pe^{-(qx)}$ where y = percent activity remaining at a given time, x = time in minutes, P = constant, q = rate constant (k), S = percent activity remaining at infinite time (final activity). In all cases, the correlation coefficient (R) was ≥0.999.

Tritium Exchange Assays. Tritium exchange assays were carried out as previously described (Seefeldt et al., 1986).

Gas Concentrations in Solution. The concentrations of gases in solution were calculated from the Bunsen coefficients (α) in water at 25 °C for O_2 (0.0283), H_2 (0.0175), N_2 (0.0143), and CO (0.0214) (Dean, 1985).

Gas Compositions. The compositions of O_2, N_2, H_2, and CO in the gas phases were verified by gas chromatography as previously described (Hyman & Arp, 1987).

Results

Reversible Inhibition by O_2. *A. vinelandii* hydrogenase was fully active in H_2 oxidation/electron acceptor reduction activity when purified under anaerobic conditions in the presence of the reductant dithionite. Progress curves of activity were linear until substrate became limiting. We wished to directly examine the possibility that O_2 might act as a rapid-equilibrium, reversible inhibitor of this hydrogenase. To do so in a H_2 oxidation/electron carrier reduction assay required that we consider the effect of reduced electron carriers produced during the assay on the concentration of O_2 in the assay. From the extinction coefficients of the electron carriers, we were able to follow the reduction of O_2 in the cuvette by the quantity of reduced dye that was reoxidized. For concentrations of O_2 of 10–50 μM, reduced benzyl viologen consumed all of the added O_2 within the mixing time of the experiment (<5 s). Reduced methylene blue, on the other hand, reacted with these concentrations of O_2 much more slowly. Complete consumption of the O_2 required approximately 1000 s. Thus, the O_2 concentration in the cuvette for the first 10 s after addition of O_2-saturated buffer was not significantly changed due to reaction with reduced methylene blue. In addition, the rate of methylene blue reoxidation (due to O_2) was insignificant compared to the rate of methylene blue reduction catalyzed by the enzyme. Thus, the slow reaction of O_2 with reduced methylene blue allowed us to directly determine the effects of O_2 on the H_2 oxidation/methylene blue reduction activity of the hydrogenase in an assay cuvette. With both benzyl viologen and methylene blue, when the consumption of O_2 by the reduced electron carrier was allowed to go to completion, the calculated concentration of O_2 was always within 97% of the quantity of O_2 added.

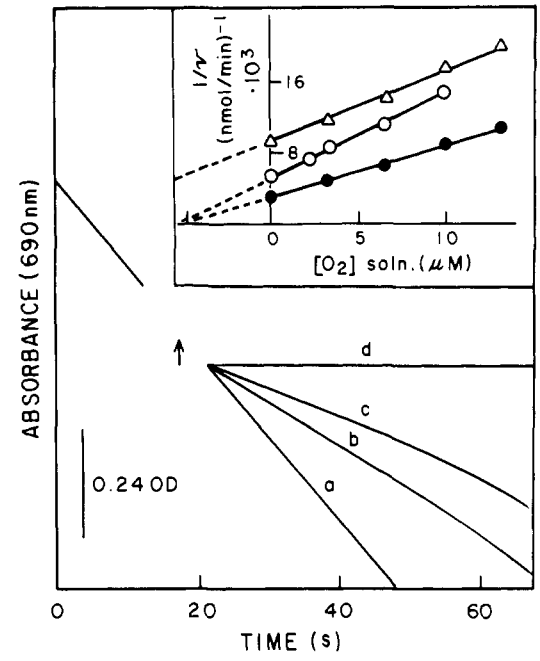


FIGURE 1: O_2 inhibition of *A. vinelandii* hydrogenase H_2 oxidation/methylene blue reduction activity. 2.5 μg of purified hydrogenase was injected into an assay cuvette that contained 6 mL of H_2-saturated 50 mM MES buffer (pH 6.0) with 150 μM methylene blue. At 15 s into the assay (arrow), O_2-saturated water was injected into the cuvette to give a final concentration of O_2 in the assay solution of 3.3 μM (b), 10 μM (c), or 40 μM (d). N_2-saturated buffer was injected into an assay cuvette (a) to give a final concentration of 40 μM N_2. At 20 s into the assay, following the addition of the O_2 or N_2, monitoring of the assay was continued. The rate of methylene blue reduction from 20 to 30 s into the assay was determined for each O_2 concentration. The reciprocal of the inhibited rate was plotted versus the O_2 concentration in the assay cuvette [(●) inset]. From this Dixon plot, the K_i was determined to be 5.5 ± 0.6 μM (r = 0.999). This series of experiments was repeated with saturating H_2 and methylene blue at 17 μM [(○) inset] and with saturating methylene blue and H_2 at 1 μM [(△) inset].

As shown in Figure 1, O_2 inhibited the H_2 oxidation/methylene blue reduction activity of *A. vinelandii* hydrogenase in a concentration-dependent process. This inhibition did not require any preincubation and was complete within the mixing time of the assay (<5 s). A plot of the reciprocal of the O_2-inhibited rates versus the O_2 concentration in solution was linear (Figure 1, inset; Dixon plot) and provided an estimate for the K_i for O_2 inhibition of 5.5 ± 0.6 μM. This experiment was repeated under conditions of saturating H_2 (688 μM in solution; K_m for H_2 = 1 μM) and lower methylene blue concentration (17 μM, K_m for methylene blue = 17 μM). A Dixon plot of the data revealed that O_2 was a noncompetitive inhibitor of hydrogenase versus oxidized methylene blue (Figure 1, inset). A similar experiment was performed but under conditions of saturating methylene blue (150 μM) and lower H_2 concentration (1 μM in solution). The Dixon plot of the data revealed that O_2 was an uncompetitive inhibitor of hydrogenase versus H_2 (Figure 1, inset).

Whereas Figure 1 demonstrates that O_2 rapidly inhibited the oxidation activity, Figure 2 shows that O_2 inhibition is also rapidly and fully reversible. In this experiment, we took advantage of the rapid (1.2×10^8 $M^{-1} \cdot s^{-1}$) and tight O_2 binding (K_d = 47 nM) properties of the soybean nodule derived protein leghemoglobin (Appleby, 1984). The addition of deoxygenated leghemoglobin to the O_2-inhibited hydrogenase under assay conditions immediately removed O_2 from solution, resulting in full recovery of H_2 oxidation activity within the mixing time of the assay (<5 s) (Figure 2).

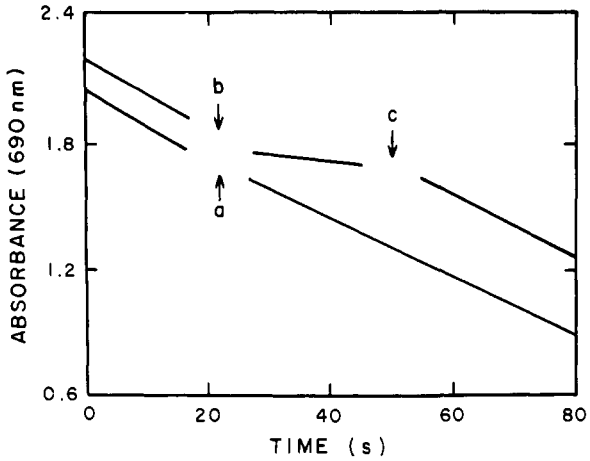


FIGURE 2: Rapid reversibility of O_2 inhibition of *A. vinelandii* hydrogenase. 2 μg of purified *A. vinelandii* hydrogenase was injected into an assay cuvette (see Experimental Procedures) to initiate the H_2 oxidation/methylene blue reduction activity assay. At 20 s into the assay, O_2-saturated water was added to one of the cuvettes to give a final O_2 concentration of 16 μM in solution (b). At 50 s into the assay, deoxygenated leghemoglobin (see Experimental Procedures) was added to this assay cuvette (140 μM final concentration) to remove the O_2 (c). The control cuvette had deoxygenated leghemoglobin (140 μM) added at 20 s into the assay (a).

All of the experiments described in the preceding section were repeated using hydrogenase still associated with the membranes. The membranes used in these experiments were incapable of consuming O_2 linked to H_2 reduction. In all cases, this membrane-associated enzyme behaved the same as the purified enzyme except that the K_i for O_2 inhibition was slightly lower for the membrane-associated enzyme (2.5 ± 0.5 μM). We conclude that O_2 acts as a rapid-equilibrium, reversible inhibitor of H_2 oxidation/methylene blue reduction activity in both the membrane-associated and purified states and is not dependent on the purification state of the enzyme.

Hydrogenases catalyze an isotope exchange reaction which does not depend on the presence of electron carriers and is presumed to involve only the H_2 activation site. We examined the possibility that O_2 could act as a reversible inhibitor of the exchange activity of *A. vinelandii* hydrogenase. Membrane-associated hydrogenase that had been activated by deoxygenation followed by addition of dithionite was assayed for $^3H_2$–H_2O exchange activity under 101 kPa N_2 or under 81 kPa N_2, 20 kPa O_2. The results presented in Figure 3 clearly demonstrate that O_2 also inhibited the exchange reaction. Removal of the O_2 by evacuation (at 15 min) reversed the inhibition and the hydrogenase recovered exchange activity. These results establish that O_2 acts as a reversible inhibitor of the exchange reaction as well as the H_2 oxidation activity linked to electron carrier reduction.

Irreversible Inactivation by O_2. In addition to the O_2-dependent, reversible inhibition described above, the active state of *A. vinelandii* hydrogenase also underwent a slower O_2-dependent, irreversible inactivation (Figure 4). In these experiments, purified enzyme that was incubated in the presence of O_2 was removed from the incubation vial and added to a H_2-purged assay cuvette containing benzyl viologen to which a small amount of dithionite was added to reduce approximately 100 μM benzyl viologen. This reduced viologen instantly consumed all of the O_2 that was carried to the cuvette with the enzyme aliquot. All of these activity assays were, therefore, done in the absence of O_2, and thus no reversible inhibition by O_2 existed. As can be seen from Figure 4, the active form of aerobically purified hydrogenase lost H_2 oxidation/benzyl viologen reduction activity in a time-dependent

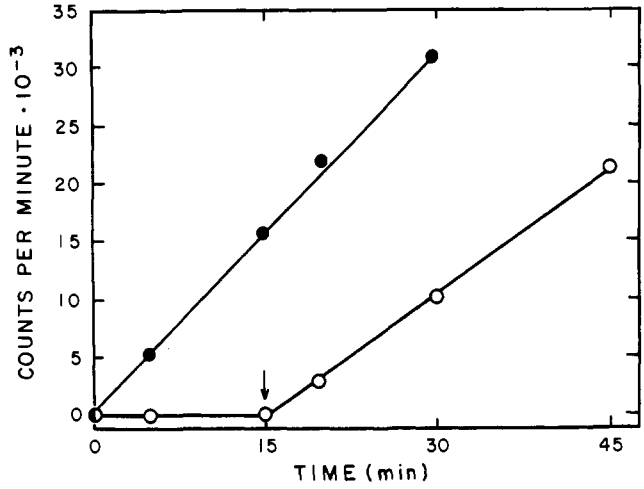


FIGURE 3: O_2-dependent, reversible inhibition of $^3H_2$–H_2O exchange activity of membrane-associated hydrogenase. *A. vinelandii* membranes were deoxygenated and activated by addition of dithionite to 2 mM. Twenty microliters of activated membranes (6 mg of protein·ml^{-1}) was added to each of two stoppered vials (8.6 mL) that contained 1 mL of 50 mM MES buffer, pH 6.0. The gas phases consisted of 101 kPa N_2 (●) or 20 kPa O_2 and 81 kPa N_2 (○). The reaction was initiated by the addition of $^3H_2$ gas (see Experimental Procedures). Aliquots (10 μL) were removed at the indicated times and counted by liquid scintillation counting and are presented as counts per minute per 10 μL. At 15 min into the assay (arrow), both samples (●, ○) were evacuated and flushed with N_2. $^3H_2$ was then immediately added back to achieve the original concentration, and aliquots were removed and counted as above at the indicated times.

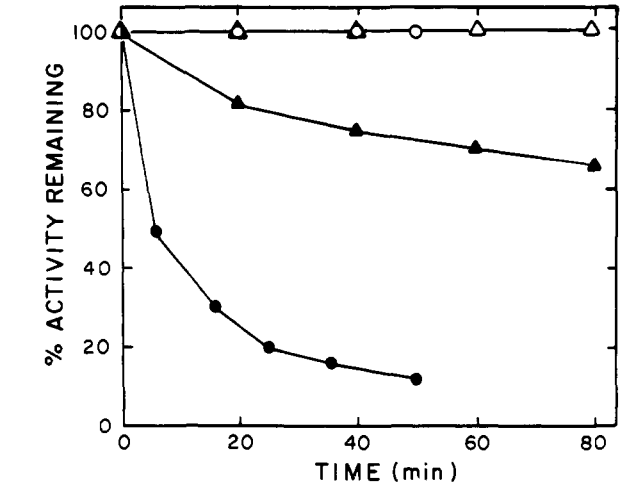


FIGURE 4: O_2-dependent, irreversible inactivation of *A. vinelandii* hydrogenase in the membrane-associated and purified states. *A. vinelandii* hydrogenase in the membrane-associated (600 μg of total protein) (▲, △) or purified (40 μg) (●, ○) state was incubated in 200 μL of buffer under 20 kPa O_2 and 81 kPa N_2. The membranes were prepared, and the enzyme was purified under aerobic conditions (Experimental Procedures). These enzyme samples were added to the incubation vial either following just deoxygenation (○, △) or following deoxygenation and activation with dithionite for 2 h (●, ▲). Aliquots (15 μL) were removed from the incubation vial at the indicated times and injected into an assay cuvette (see Experimental Procedures) that contained either methylene blue (△, ▲) or benzyl viologen (○, ●) as the electron acceptor. The percentage of the initial activity remaining at the indicated times was plotted against the time of incubation in O_2.

manner. The same rate and extent of inactivation was observed for aerobically purified, activated enzyme and anaerobically purified active enzyme. A semilog plot of percentage activity remaining versus time was linear, indicating that the process was first order. This process was characterized by a rate constant (k) of 0.12 ± 0.02 min^{-1}. It is noteworthy that

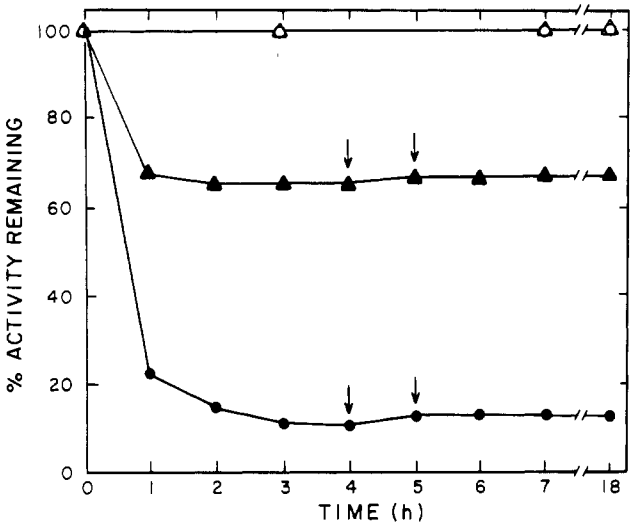


FIGURE 5: Irreversibility of O_2-inactivated hydrogenase in the membrane-associated and purified states. *A. vinelandii* hydrogenase was prepared under anaerobic conditions in the presence of 2 mM dithionite in the purified (40 μg) (○, ●) or membrane-associated (600 μg of total protein) (△, ▲) states and was added to the appropriate stoppered vials to initiate the incubations. The vials contained the following gas phases equilibrated with 200 μL of buffer (Experimental Procedures): (●, ▲) 20 kPa O_2 and 81 kPa N_2; (○, △) 101 kPa N_2. At the indicated times, an aliquot (15 μL) was removed from the incubation vial and injected into a H_2 oxidation/dye reduction cuvette (see Experimental Procedures). At 4 h (arrow), the incubation vials were evacuated and purged with H_2. At 5 h (arrow), dithionite was added to give a final concentration of 2 mM. The percent of the initial activity remaining was plotted against the time of incubation.

the enzyme activity did not proceed to 0% activity but instead approached 10% remaining activity (Figure 5). The susceptibility of hydrogenase still associated with membranes to inactivation by O_2 was also investigated. Hydrogenase associated with membranes did not reduce benzyl viologen, so all activity assays with membranes utilized methylene blue as the electron acceptor. In these cases, the small amounts of O_2 transferred with the enzyme to the reaction mixture were consumed during the first 60 s of the assay, after which the progress curves of methylene blue reduction were linear. Maximal rates were taken from the linear portion of the progress curves. Activated membranes exhibited an O_2-dependent, irreversible inactivation of hydrogenase activity (Figure 4). A semilog plot of percent activity remaining versus time was linear, indicating that the process was first order, although the rate constant ($k = 0.015 \pm 0.001$ min^{-1}) and residual activity were different from purified enzyme. The inactivation of the hydrogenase in the membrane also did not go to 0% activity but instead approached 60% remaining activity (Figure 5). Membranes that had been fully activated by deoxygenation followed by incubation in 1% H_2 retained full activity even after removal of the H_2 by evacuation. However, hydrogenase activity in these membranes after H_2 was removed was irreversibly inactivated by O_2 with similar kinetics as observed for the membrane-associated enzyme activated with dithionite. This demonstrated that O_2-dependent, irreversible inactivation was not a consequence of dithionite activation alone. Extended treatment of O_2-inactivated hydrogenase, either purified or membrane-associated, in the absence of O_2 and in the presence of H_2 and dithionite did not result in reactivation of the enzyme, thus confirming that the O_2-dependent inactivation was irreversible (Figure 5).

The rate of inactivation of anaerobically purified hydrogenase was the same under gas phases containing 101, 20, 1, and 0.5 kPa O_2 (Table I). Thus, the rate of inactivation was saturated even at 0.5 kPa O_2 (6 μM in solution). We confirmed that the dithionite carried over with the enzyme was consumed by O_2 within 15 s at partial pressure of O_2 as low as 0.5 kPa. At lower concentrations of O_2, however, the dithionite was not consumed as rapidly, and thus experiments at lower O_2 concentrations could not be performed accurately.

Table I: O_2 Inactivation of Purified *A. vinelandii* Hydrogenase

treatment[a]	% activity remaining[b]	treatment[a]	% activity remaining[b]
101 kPa N_2	100	pH 6.0[c]	17
101 kPa O_2	24	pH 7.5	26
20 kPa O_2	26	pH 9.0	37
1 kPa O_2	25	1 M NaCl	25
0.5 kPa O_2	25	30 mM mercaptoethanol	24
2 kPa H_2	96	superoxide dismutase/	24
0.4% Emulgen 913	25	catalase[d]	

[a] Incubations were as described under Experimental Procedures with a gas phase of 20 kPa O_2 and the remainder N_2 to bring to 101 kPa unless otherwise stated. [b] The percent activity remaining (H_2 oxidation/benzyl viologen reduction) is after 35-min incubation under the noted conditions. [c] The buffer used for incubations at pH 6.0, 7.5, and 9.0 was 20 mM each MES, HEPES, and Tris. [d] Superoxide dismutase and catalase were at 0.5 mg·mL^{-1} each.

Protection of Hydrogenase from Reversible Inhibition and Irreversible Inactivation by O_2. There have been various reports in the literature of agents which provided protection of hydrogenases from interaction with O_2 [see Adams et al. (1981) and Cammack et al. (1988) for reviews]. We have examined several of these agents for their ability to protect *A. vinelandii* hydrogenase from reversible inhibition and irreversible inactivation by O_2. Table I shows that the presence of detergent, 1 M NaCl, 30 mM mercaptoethanol or 0.5 mg/mL each of superoxide dismutase and catalase, had no effect on the rate or extent of irreversible inactivation of *A. vinelandii* hydrogenase by O_2. Likewise, none of these agents had any effect on the irreversible inactivation by O_2 of the membrane-associated hydrogenase. In addition, the presence of these agents did not change the K_i for the rapid-equilibrium, reversible inhibition by O_2 of this hydrogenase (data not shown).

The substrate H_2 has been examined as a potential protectant from irreversible inactivation by O_2 for a few hydrogenases, and in no case was any significant protective effect observed. In fact, Schink and Probst (1981) established that the presence of H_2 actually accelerates the inactivation of the membrane-bound hydrogenase from *Alcaligenes eutrophus* H16 by O_2. In contrast, we found that the presence of low concentrations of H_2 provided nearly complete protection from O_2-dependent, irreversible inactivation of *A. vinelandii* hydrogenase (Table I). Figure 6 shows the concentration dependence of the H_2 protection from inactivation by O_2 of *A. vinelandii* hydrogenase. The presence of H_2 in solution at nearly the same concentration as the K_m for H_2 (0.2 kPa H_2, 1 μM in solution) provided almost complete protection from inactivation by O_2, while the presence of H_2 at concentrations less than the K_m provided decreasing levels of protection from O_2-dependent inactivation. H_2 also protected hydrogenase associated with membranes from O_2-dependent inactivation over a similar concentration range. At saturating concentrations of H_2, complete protection from inactivation by O_2 was provided for at least 18 h.

In the experiments described above, H_2 was present at the initiation of the incubation where it provided concentration-dependent protection from irreversible inactivation by O_2. The experiment shown in Figure 7 demonstrated that the addition

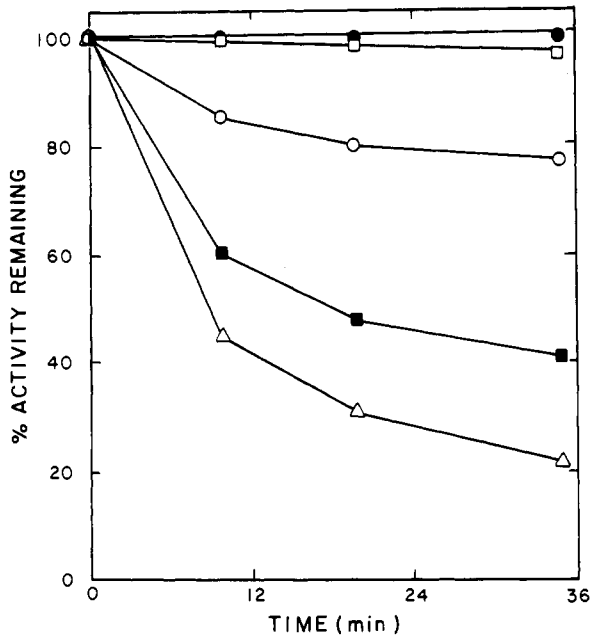


FIGURE 6: Concentration dependence of H_2 protection from O_2-dependent, irreversible inactivation of purified *A. vinelandii* hydrogenase. Stoppered vials were prepared with 100 μL of buffer and a gas phase of 20 kPa O_2 and the following gases: (●) 81 kPa H_2; (□) 80.8 kPa N_2 and 0.2 kPa H_2; (○) 81 kPa N_2 and 0.04 kPa H_2; (■) 81 kPa N_2 and 0.007 kPa H_2; (△) 81 kPa N_2. Anaerobically purified hydrogenase (40 μg) was added to each vial to initiate the incubation. At the indicated times, an aliquot (10 μL) was removed from the incubation vial and injected into an H_2 oxidation/benzyl viologen reduction assay cuvette (see Experimental Procedures). The percent of initial activity remaining is plotted versus the time of incubation.

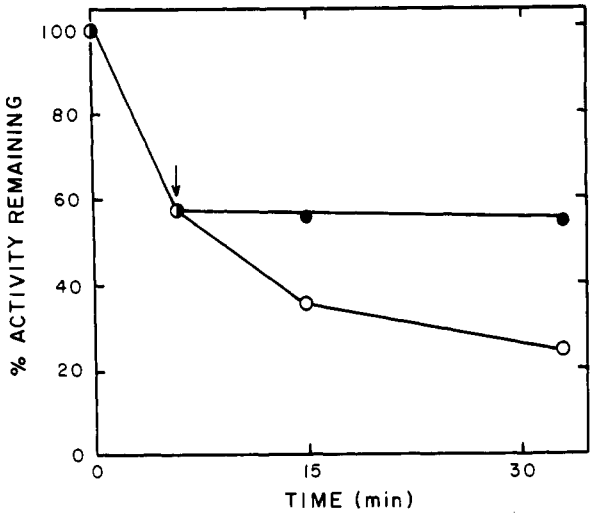


FIGURE 7: H_2 protection from O_2-dependent, irreversible inactivation of *A. vinelandii* hydrogenase. Two stoppered incubation vials were prepared, each with 100 μL of buffer and a gas phase of 20 kPa O_2 and 81 kPa N_2. Anaerobically purified *A. vinelandii* hydrogenase (40 μg) was added to each vial to initiate the incubation. At 6 min, an aliquot (10 μL) was removed from each vial and assayed for H_2 oxidation/benzyl viologen reduction activity (see Experimental Procedures). At 6 min (arrow), H_2 was added as an overpressure to one vial (●) to give a final H_2 concentration of 1 kPa. The same volume of N_2 was added to the other vial (○) at 6 min. Aliquots were removed from the vials at the indicated times and assayed for H_2 oxidation/benzyl viologen reduction activity. The precentage of initial activity remaining is plotted against the time of incubation.

of H_2 to an incubation vial during the course of O_2-dependent, irreversible inactivation immediately halted any further inactivation and did not reactivate the fraction of enzyme that had already been inactivated.

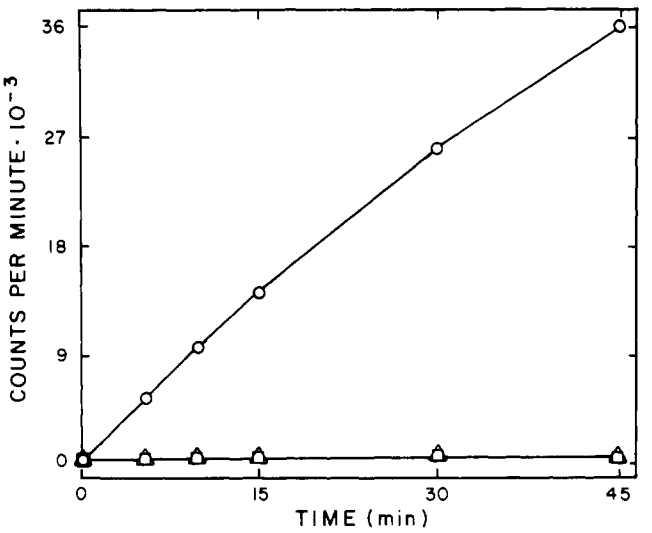


FIGURE 8: H_2 protection from O_2-dependent inactivation of hydrogenase in the presence of $^3H_2$. Stoppered vials were prepared with a gas phase of 101 kPa N_2 (○), 20 kPa O_2 and 0.07 kPa H_2 (△), or 20 kPa O_2 (□). All vials were adjusted to 101 kPa with N_2. $^3H_2$ gas was added to each vial (see Experimental Procedures), and the vials were allowed to equilibrate for 5 min. Anaerobically purified hydrogenase (10 μg) was added to each vial to initiate the reaction. At the indicated times, an aliquot (10 μL) was removed from each vial and was counted by liquid scintillation counting. The counts per minute for 10 μL of sample is plotted against the time of incubation.

We wished to examine whether H_2 was being consumed by the enzyme during H_2 protection from irreversible inactivation by O_2. Gas chromatographic analyses of the gas phases of incubation vials such as those described in Figure 6 at the initiation of the incubation and at the end of the incubation revealed no detectable changes in the H_2 or O_2 concentrations over the course of the experiment. This suggested that if the H_2 was consumed during the course of H_2 protection from O_2 inactivation, the rate must be extremely low and thus the concentration change could be below the detection limits of the gas chromatograph. The experiment summarized in Figure 8 addresses this possibility of a low rate of consumption of H_2 by the hydrogenase during H_2 protection by taking advantage of the high sensitivity of scintillation counting of ^3H_2O. In this experiment, $^3H_2$ was mixed with $^1H_2$ and served as a marker for H_2 oxidation to H_2O. These experiments would detect both the exchange reaction and the oxidation of H_2. As shown (Figure 8), active hydrogenase catalyzed the exchange of H_2 and $^3H_2$ to H_2O and ^3H_2O where ^3H_2O is counted by scintillation counting. Under identical conditions, except where O_2 was included, the enzyme did not catalyze any detectable consumption of $^3H_2$. An aliquot removed from this latter incubation vial at 45 min was also assayed for H_2 oxidation/benzyl viologen reduction activity and showed >96% activity remaining. Thus, under these conditions, the enzyme was fully protected by H_2 from irreversible inactivation by O_2, yet the H_2 was not consumed or exchanged as part of this protection.

Effect of CO on Irreversible Inactivation by O_2 and Protection by H_2. CO has been shown to be a competitive inhibitor versus H_2 for several hydrogenases, including the hydrogenase from *A. vinelandii* where the K_i was found to be 25 μM (Hyman & Arp, 1987). In addition, CO was shown to protect *A. vinelandii* hydrogenase from inhibition by the active-site-directed inhibitor C_2H_2 (Hyman & Arp, 1987). We have examined the effects of CO on the O_2-dependent, irreversible inactivation of *A. vinelandii* hydrogenase and on the protection by H_2. Figure 9 shows that CO had no effect on

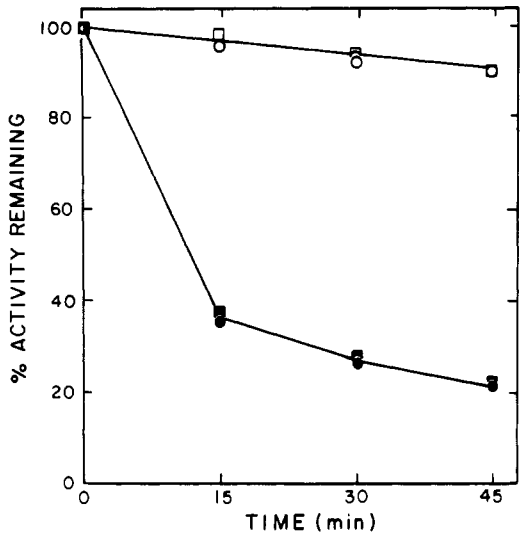


FIGURE 9: Effect of CO on the O_2-dependent, irreversible inactivation and H_2 protection of hydrogenase. Stoppered incubation vials were prepared with 100 μL of buffer and a gas phase of 20 kPa O_2 and 81 kPa CO (○, ●) or 20 kPa O_2 and 81 kPa N_2 (□, ■). Two vials also contained 0.2 kPa H_2 (□, ○). Anaerobically purified hydrogenase (10 μg) was added to each vial to initiate the incubations. At the indicated times, an aliquot (10 μL) was removed from each vial and assayed for H_2 oxidation/benzyl viologen reduction activity. The percent of initial activity remaining is plotted against the time of incubation.

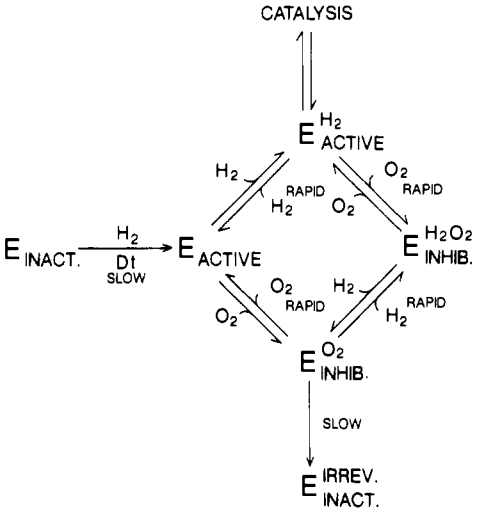
the rate or extent of irreversible inactivation by O_2 even under conditions of saturating CO (81 kPa in the gas phase, 0.7 mM in solution). In addition, it was clear that the H_2 protection from O_2 inactivation (K_m concentrations of H_2, 1 μM in solution) was not affected by the presence of saturating CO.

Discussion

As outlined in the introduction, the interactions of O_2 with hydrogenases in general appear to be rather complex. In the present work, we have characterized both a reversible and an irreversible effect of O_2 on the hydrogenase from *A. vinelandii*. We propose a model (Scheme I) that outlines the states of this hydrogenase and the effects of O_2. This model provides a basis for the discussion that follows.

Reversible Inhibition by O_2. We demonstrated that *A. vinelandii* hydrogenase, both in the purified and in the membrane-associated states, was susceptible to a rapid-equilibrium, reversible inhibition by O_2 (Figures 1 and 2). This inhibition can be compared to that reported for the immunologically related hydrogenases purified from *B. japonicum* and *A. eutrophus* H16. *B. japonicum* hydrogenase was shown to be inhibited by O_2 with a K_i of 10 μM; this inhibition was uncompetitive versus H_2 and noncompetitive versus methylene blue (Arp & Burris, 1981). The results presented here for *A. vinelandii* hydrogenase are similar to those reported for *B. japonicum* hydrogenase. One difference is that in the present study, O_2 inhibition was immediate (within 5 s), while it was reported that *B. japonicum* hydrogenase required 1-min preincubation in O_2 prior to addition of H_2 to observe maximal inhibition by O_2. The membrane-bound hydrogenase from *A. eutrophus* H16 was also inhibited by O_2, but this inhibition was described as competitive versus H_2 with a K_i of 17 μM (Schink & Probst, 1980). This contrasts with the uncompetitive inhibition between O_2 and H_2 observed for *A. vinelandii* hydrogenase presented here. Perhaps this reflects different mechanisms of O_2 inhibition for these two hydrogenases. It should be noted, however, that the study of *A. eutrophus* hydrogenase utilized manometric assays and that

Scheme I: Model for the Interaction of O_2 and H_2 with *A. vinelandii* Hydrogenase



it was assumed that O_2 was immediately consumed by reduced methylene blue. Similar inhibition properties might be revealed if the *A. eutrophus* hydrogenase were reanalyzed for O_2 inhibition directly, especially considering the fact presented here that small amounts of O_2 are slowly consumed by reduced methylene blue. The two previous studies of O_2 inhibition of these related hydrogenases only examined the effect of O_2 on H_2 oxidation linked to the reduction of the electron acceptor methylene blue (Arp & Burris, 1981; Schink & Probst, 1980). It seemed possible that O_2 inhibition might affect the H_2 oxidation activity of the enzyme without affecting the H_2 binding site. To address this possibility, we examined the effect of O_2 on the exchange activity of the enzyme. This activity is presumed to involve only the H_2 binding site of the enzyme. As shown in Figures 3 and 8, O_2 also reversibly inhibited the exchange reaction. Thus, we have shown that O_2 acts as a rapid-equilibrium, reversible inhibitor of both the exchange and H_2 oxidation activities of *A. vinelandii* hydrogenase. This inhibition is represented by the interconversion between the active state (E_{active}) and the reversibly inhibited state ($E^{O_2}_{inhib}$) in Scheme I.

In considering a potential mechanism of O_2 inhibition of *A. vinelandii* hydrogenase, it is necessary to consider the redox centers of this enzyme. Elemental analysis of purified *A. vinelandii* hydrogenase reported 6.6 Fe and 0.7 Ni per molecule (Seefeldt & Arp, 1986). Considering these data and by analogy to other Ni- and Fe-containing dimeric hydrogenases, it seems likely that the *A. vinelandii* hydrogenase contains one nickel center and at least two 4Fe–4S centers. While the exact role of Ni and Fe–S centers in catalysis is not known for any hydrogenase, it is accepted that some or all of the metal centers have central roles in the catalytic mechanism. Given the known metal binding and oxidizing properties of O_2, a likely site of O_2 binding for O_2 inhibition would be one or more of the metal centers of the hydrogenase. The fact that O_2 is not a competitive inhibitor of activity versus the substrates H_2 and methylene blue would suggest that O_2 binds at a site other than the H_2 and methylene blue binding sites. However, O_2 did inhibit the exchange reaction, thought to involve only the H_2 binding site. These results could be explained in several ways. One possibility is that O_2 may still bind to the same metal center as H_2 binds to, although, since the two are not competitive, they would not bind mutually exclusively. This mechanism could explain the O_2 inhibition of the exchange reaction. The present data could also be interpreted by a

mechanism whereby the exchange reaction involved more than one metal center and that O_2 binding to a second metal center could still inhibit the exchange reaction. It seems likely that O_2 inhibition may be more complex than simple binding and might involve an oxidation of a metal center. In this case, the O_2 would bind to and oxidize one or more metal center(s), rendering the enzyme inhibited. Removal of the O_2 would allow the enzyme to be rapidly reduced by H_2 present in the assay and thus reactivated.

Irreversible Inactivation by O_2. In addition to the rapid-equilibrium, reversible inhibition of *A. vinelandii* hydrogenase by O_2, we report a slower, O_2-dependent, irreversible inactivation of this hydrogenase (Figures 4 and 5). This irreversible inactivation is represented by the conversion from $E_{inhib}^{O_2}$ to E_{inact}^{irrev} in Scheme I. The irreversible inactivation was clearly dependent on the activation state of the enzyme. As reported previously, the *A. vinelandii* hydrogenase can be purified under aerobic conditions in a reversibly inactive state (E_{inact}) that is relatively stable toward O_2 (Seefeldt & Arp, 1987). This state of the enzyme (E_{inact}) can be converted to the active state (E_{active}) by removal of O_2 followed by incubation in dithionite or H_2 (Hyman et al., 1988). Once activated, the enzyme becomes susceptible to O_2 inactivation just like the anaerobically prepared enzyme, suggesting that the active state (E_{active}) of the enzyme behaves the same toward O_2 regardless of the history of the enzyme.

During the course of irreversible inactivation by O_2, the enzyme is in the reversibly inhibited state ($E_{inhib}^{O_2}$) and is fully inhibited. Thus, it can be concluded that irreversible inactivation by O_2 occurs from the O_2-inhibited state ($E_{inhib}^{O_2}$). The model (Scheme I) would predict that this O_2-dependent inactivation should be first order. The data reveal that this inactivation is a first-order process for both purified and membrane-associated states. It seems likely that irreversible inactivation by O_2 involves one or more of the metal centers in the enzyme. O_2 oxidative destruction of Fe–S centers has been described (Petering et al., 1971) and could be the mechanism of O_2-dependent, irreversible inactivation of *A. vinelandii* hydrogenase. As shown in Figure 5, the hydrogenase in both the purified and membrane-associated states was not fully inactivated even after 18-h incubation under O_2. One possible explanation would be that O_2 oxidatively destroys a portion of the centers involved in catalysis (e.g., conversion from a 4 Fe–4 S to a 3 Fe–*X* S center), resulting in a reduced rate of electron transfer through an alternate pathway within the enzyme. The difference between purified and membrane-associated enzyme is unknown. Whatever the cause of this lack of complete inactivation, it provides further evidence differentiating this process from the O_2 inhibition, which is immediate and complete even at low concentrations of O_2.

O_2-dependent, irreversible inactivation of other hydrogenases has been studied, and half-lives varying from several minutes to several hours have been reported (Cammack et al., 1988). The half-life of the *A. vinelandii* hydrogenase reported here of 5.9 min is comparable to the shortest half-lives reported for hydrogenases (2 min) (Cammack et al., 1988). This inactivation appears similar to that previously reported for a partially purified preparation of hydrogenase from *A. vinelandii* (Kow & Burris, 1984) although in that case the inactivation kinetics were not characterized. There are numerous reports in the literature examining agents as potential protectants of hydrogenase from inactivation by O_2 (Adams et al., 1981; Cammack et al., 1988). We have examined many of these agents for their ability to protect *A. vinelandii* hydrogenase from irreversible inactivation by O_2. As summarized in Table I, 1 M NaCl, 30 mM mercaptoethanol, and superoxide dismutase/catalase had no effect on the rate or extent of irreversible inactivation of *A. vinelandii* hydrogenase by O_2. These results suggest that the inactivation is not the result of oxidation of a catalytically important sulfide on the enzyme and that radical species of oxygen are not responsible, as is clearly the case for the flavin-containing, soluble hydrogenase from *A. eutrophus* (Schneider & Schlegel, 1981).

The substrate H_2 has also been examined as a potential protective agent from inactivation by O_2 for the hydrogenases from *Desulfovibrio vulgaris* (Mayhew et al., 1978), *Chlamydomonas reinhardii* (Erbes et al., 1979), *A. eutrophus* (membrane-bound and soluble) (Schink & Probst, 1980; Schneider & Schlegel, 1981), *Clostridium pasteurianum* (Khan et al., 1981), *Chromatium vinosum* (Kakuno et al., 1978), and *Alcaligenes latus* (Pinkwart et al., 1983). In no case was protection from O_2 inactivation by H_2 observed. Therefore, we were surprised to find that H_2 was a very potent protectant from inactivation by O_2 of *A. vinelandii* hydrogenase. As summarized in Figure 6, the presence of near-K_m concentrations of H_2 in solution provided nearly complete protection from irreversible inactivation by O_2. At concentrations of H_2 substantially less than K_m, a concentration dependence of the H_2 protection was observed. At concentrations of H_2 higher than K_m, the enzyme was completely protected from inactivation by saturating concentrations of O_2 for at least 20 h. This represents the first reported case of H_2 protection from O_2 inactivation. If H_2 protection from O_2 inactivation was a result of H_2 maintaining the enzyme in a partially reduced state, then perhaps O_2 could serve as the oxidant to couple to H_2 oxidation resulting in H_2 consumption. This possibility was inconsistent, however, with the fact that the hydrogenase should be fully inhibited in the presence of O_2 ($E_{inhib}^{O_2}$), even with H_2 present. Indeed, the experiment shown in Figure 8 confirms that H_2 is not being consumed during H_2 protection from O_2 irreversible inactivation. H_2 must bind to the O_2-inhibited state ($E_{inhib}^{O_2}$), taking the enzyme to a new state ($E_{inhib}^{O_2H_2}$) that, while inhibited by O_2, is protected from irreversible inactivation by O_2. Thus, irreversible inactivation of hydrogenase (E_{inact}^{irrev}), as shown in Scheme I, can only occur from the O_2-inhibited state ($E_{inhib}^{O_2}$). Figure 7 provides further evidence for this model, revealing that H_2 can bind to the O_2-inhibited state ($E_{inhib}^{O_2}$), preventing any further inactivation by O_2 by taking the inhibited enzyme to the inhibited, but stable state ($E_{inhib}^{O_2H_2}$). To complete the model presented in Scheme I, it is known that active hydrogenase (E_{active}) can bind H_2 since it can catalyze the exchange reaction ($E_{active}^{H_2}$). Addition of O_2 to this state results in the conversion to the O_2-stable, but inactive state ($E_{inhib}^{H_2O_2}$). The results provided here would indicate that H_2 can bind to the O_2-inhibited state but that H_2 cannot undergo exchange or oxidation. One possible explanation would be that H_2 is binding to its normal binding site but is unable to be cleaved due to the presence of O_2 bound at the same or a different metal center. The presence of bound H_2, while it did not prevent O_2 inhibition, did prevent O_2 irreversible inactivation, thus providing further evidence that O_2 inhibition and O_2 inactivation are separate processes.

CO is known to be a competitive, reversible inhibitor versus H_2 for the *A. vinelandii* hydrogenase and is thus presumed to bind mutually exclusively with H_2 at the normal H_2 binding site. CO also inhibits the exchange reaction (Seefeldt and Arp, unpublished results). CO was able to compete with C_2H_2, just like H_2 could, confirming that acetylene was active site directed (Hyman & Arp, 1987). We examined the effects of CO on

the irreversible inactivation by O_2 of *A. vinelandii* hydrogenase. Erbes et al. (1979) observed that hydrogenase from *C. rheinhardii* could be protected from O_2 inactivation by CO and from this concluded that O_2 inactivation involved the active (H_2) site, although H_2 did not protect from O_2 inactivation. As shown in Figure 9, the presence of saturating CO did not protect *A. vinelandii* hydrogenase from O_2-dependent, irreversible inactivation. This is in contrast to the nearly complete protection from O_2 inactivation afforded by traces of H_2. In addition, saturating CO did not prevent low (1 μM) concentrations of H_2 from protecting the enzyme from O_2 inactivation. This suggests that binding of O_2 which leads to irreversible inactivation is not occurring at the H_2 binding site, even though H_2 can protect from the inactivation. Thus, H_2 protection from O_2 inactivation is more than simple competition for the H_2 activation site. This would be consistent with the proposal that H_2 binds to its normal H_2 site and stays bound until the O_2 inhibition is removed by removal of O_2. These data are also consistent with H_2 binding to a site other than to the H_2 activating site. This could be a regulatory site, as has been suggested from the activation work on *D. gigas* hydrogenase (Cammack et al., 1988).

The kinetic characterization of the effects of O_2 on *A. vinelandii* and the model presented here should provide the necessary information to allow examination by physical techniques, such as EPR, to determine the oxidation states of the metal centers in this enzyme in each of the states proposed. In this way, a comprehensive mechanism of action of O_2 and the properties of the redox centers in this enzyme should be elucidated.

Acknowledgments

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Registry No. O_2, 7782-44-7; H_2, 1333-74-0; hydrogenase, 9027-05-8; methylene blue, 61-73-4.

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