The Physical and Catalytic Properties of Hydrogenase II of
Clostridium pasteurianum

A COMPARISON WITH HYDROGENASE I*

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Hydrogenase II of Clostridium pasteurianum is a monomeric protein of \( M_r = 53,000 \) containing 8 iron and 8 acid-labile sulfide atoms/mol. It is distinct from hydrogenase I from the same organism \( (M_r = 60,000 12 \text{ Fe and } 12 \text{ S}^2-/\text{mol}) \). Metal analyses showed that neither hydrogenase contains nickel or any other metals in significant amounts. The iron atoms of hydrogenase II resisted chelation by 2,2'-bipyridyl but all were susceptible when the enzyme was treated with ferricyanide. Core extrusion indicated the presence of two \( (4\text{Fe}-4\text{S}) \) clusters in hydrogenase II and EPR spectroscopy showed two distinct paramagnetic species which could be interpreted as one \( (4\text{Fe}-4\text{S})^{2+} \) and one \( (4\text{Fe}-4\text{S})^{1+} \) per molecule. The absorption coefficient of \( H_2 \)-reduced hydrogenase II at 420 nm was 23,000 \( M^{-1} \text{ cm}^{-1} \) with a \( A_{420}/A_{276} \) ratio of 0.27. There were large differences between hydrogenase I and hydrogenase II in the absorption spectra of the air-oxidized, \( H_2 \)-reduced, and dithionite-reduced forms of the enzymes. Hydrogenase II catalyzed \( H_2 \) evolution with methyl viologen or ferredoxin as the electron carrier, and \( H_2 \) oxidation with methylene blue or methyl viologen as the electron acceptor. Apparent \( K_m \) values were determined for all these reactions with both hydrogenases. Hydrogenase II is a relatively inactive enzyme, except in the reduction of methylene blue by \( H_2 \). The \( pH \) dependencies of \( H_2 \) oxidation were similar for both hydrogenases but were very different in \( H_2 \) evolution. The activation energy values were much higher for \( H_2 \) catalysis by hydrogenase II than for hydrogenase I. The two hydrogenases have the same sensitivity to inactivation by \( O_2 \) but differ in their sensitivity to metal-chelating reagents and to CO. Hydrogenase I is more readily inhibited by CO but hydrogenase II binds CO irreversibly. From the above data, a mechanism is proposed to account for the observed differences in the catalytic activities of hydrogenase I and hydrogenase II.

The anaerobic \( N_2 \)-fixing bacterium Clostridium pasteurianum produces large amounts of \( H_2 \) as an end product of metabolism. These saccharolytic organisms metabolize carbohydrates to organic acids, \( H_2 \), and \( CO_2 \) and because they lack an electron transport chain, they obtain ATP by substrate level phosphorylation. The enzyme hydrogenase (EC class 1.12) which catalyzes the reversible activation of \( H_2 \) has been purified from this organism and has been characterized (Chen and Mortenson, 1974; Chen et al., 1976). It is an iron-sulfur protein of \( M_r = 60,000 \) containing 12 iron atoms and 12 acid-labile sulfide atoms/mol. A second hydrogenase species was recently discovered in C. pasteurianum Chen and Blanchard (1978), and we have now obtained this enzyme in a pure form. The latter hydrogenase, which we have termed hydrogenase II, is also an iron sulfur protein but it has \( M_r = 53,000 \) and contains 8 iron and 8 acid-labile sulfide atoms/mol. The aim of the present work was to characterize hydrogenase II and compare its properties with those of hydrogenase I.

In the last few years, hydrogenases have been purified from a variety of microorganisms but a comparison of their properties reveals only one common feature: all are iron-sulfur proteins. Other than this, they differ considerably in molecular composition, metal content, specific activity, and their sensitivity to inactivation by \( O_2 \) (for review, see Adams et al., 1981). In addition, two recent developments in the field of iron-sulfur proteins in general and in the area of hydrogenases in particular have made a detailed characterization of the clostridial hydrogenases even more crucial. The first is the discovery of a new type of iron-sulfur center in proteins, the \( [3\text{Fe}-3\text{S}] \) cluster first identified in ferredoxins, from Azotobacter vinelandii (Emptage et al., 1980) and Desulfovibrio gigas (Huynh et al., 1980). A 3Fe center has now been reported in the hydrogenases isolated from Chromatium (Albracht et al., 1982b) and Desulfovibrio desulfuricans (Kruger et al., 1982). The other important finding is that many hydrogenases contain nickel atoms in addition to iron and inorganic sulfur as part of the active enzyme (see Kruger et al., 1982, and references therein). Spectroscopic studies have indicated that nickel functions as an additional redox center (Cammack et al., 1982; LeGall et al., 1982). As yet, it is not known if nickel is obligatory for \( H_2 \) catalysis by hydrogenase or the role that nickel plays in catalysis.

We have previously shown that hydrogenase II of C. pasteurianum is extremely active in \( H_2 \) oxidation with methylene blue as the electron acceptor but that it catalyzes \( H_2 \) evolution from reduced methyl viologen at very low rates. On the other hand, hydrogenase I catalyzes both reactions at extremely high rates (Chen and Mortenson, 1974) and is one of the most active hydrogenases so far isolated (see Adams et al., 1981). We therefore investigated the catalytic properties of hydrogenase II with the hope of gaining some insight into the mechanistic differences between the two clostridial hydrogenases and perhaps of \( H_2 \) catalysis in general. In addition, we report some physical characteristics of hydrogenase II which are compared with those of hydrogenase I and of other well studied hydrogenases.

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Hydrogenase I1 of Clostridium pasteurianum

MATERIALS AND METHODS

Materials—All reagents and chemicals were of the highest purity commercially available. Hydrogenase I, hydrogenase II, and ferredoxin were purified from N2-grown C. pasteurianum W5 cells as described previously.

Enzyme Assays—Hydrogenase activity was routinely determined spectrophotometrically by measuring the reduction of methylene blue as described by Adams and Mortenson. The assay mixture contained methylene blue (0.05 mM) in 50 mM Tris/HCl, pH 8.0, under H2 at 30°C. The rate of hydrogenase activity catalyzes the reduction of 1 μmol of methylene blue/min under these conditions, which is equivalent to 1 amol of H2 oxidized/min. For methyl viologen, the reaction was measured at 600 nm, and an absorption coefficient of 12,000 M⁻¹ cm⁻¹ was assumed for the semiquinone form. Results are also expressed as micromoles of H2 oxidized/min, equivalent to 2 μmol of methyl viologen reduced/min. For the determination of the Ke₃ values for H2-degassed assay cuvettes were flushed with various H2/Ar mixtures and the pH was quantitated by removing samples from the gas phase and analyzing by gas chromatography. A solubility coefficient of 0.0189 was used to determine the concentration of dissolved H2 (see Vignais et al., 1982). Hydrogenase activity was also measured by H2 production with methyl viologen (1 mM) as the electron carrier and sodium dithionite (20 mM) as the electron donor in 50 mM Tris/HCl, pH 8.0. Activity is expressed as micromoles of H2 evolved/min. For the assay of hydrogenase I, all reaction mixtures contained bovine serum albumin (1 mg/ml) in both assay systems.

Metal Analyses—To ascertain the presence of any other metals in hydrogenase II, the enzyme was examined by plasma emission spectroscopy. This gave a 32-element analysis (two nonmetals). Results from three determinations with different hydrogenase II preparations gave values of 8.0 ± 0.48 g atoms of iron/53,000 g of protein. Analyses for Ni, Mo, Cu, Zn, Mn, and Se were all less than 0.1 g atoms/mol of enzyme. Similar results were obtained with hydrogenase I: that is, iron was the only metal present in molar amounts equivalent to or greater than the moles of protein. Values of 11.4 ± 0.51 g atoms of Fe/mol of protein were found; with Ni, less than 0.1 g atoms/mol were found.

The iron atoms of hydrogenase II were resistant to chelation by 2,2'-bipyridyl under anaerobic conditions indicating that all the iron is part of the active enzyme. However, the addition of the protein-denaturing agent sodium dodecyl sulfate (1%, w/v) resulted in the immediate formation of the ferrous-2,2'-bipyridyl complex equivalent to the release of 7.8 ± 0.3 g atoms of iron/mol of enzyme (from three determinations). All the hydrogenase activity (both H2 evolution and H2 uptake) was lost immediately upon the addition of sodium dodecyl sulfate.

Effect of Potassium Ferricyanide—Potassium ferricyanide is a commonly used oxidant in biological redox systems, and its effect on the activity and integrity of hydrogenase I1 was investigated. When the enzyme was treated with a 200-fold excess of ferricyanide under anaerobic conditions, no hydrogenase activity could be detected after a 5-min incubation period in either the H2 uptake or the H2 evolution assay. Sodium dithionite and 2,2'-bipyridyl were then added as described under “Materials and Methods.” The immediate red coloration showed that iron had been released from the enzyme by the ferricyanide treatment. Quantitation gave values of 7.92 ± 0.25 g atoms of iron/mol of hydrogenase II indicating that ferricyanide treatment is nonselective; all the iron atoms were released. Control experiments in the absence of hydrogenase showed that potassium ferricyanide contains 0.77% free iron.

Active Site Extrusion—Kurtz (1982) reported a new method for the extrusion of [2Fe-2S] clusters from ferredoxins which involved treating the protein with thiophenol in DMF (6-17%, v/v)-Triton X-100 (5%, v/v) mixtures. Advantages over the previous method (see Gillum et al., 1977) include avoidance of toxic HMBA, lower concentrations of organic solvents, and for spinach ferredoxin (2Fe) a decreased extrusion time. To discern the nature of the active site of hydrogenase I1, the new extrusion procedure was first investigated with C. pasteurianum ferredoxin which contains two [4Fe-4S] centers (Que et al., 1975). Fig. 1 shows the results of one experiment using a final solvent composition of 79:14:7 (buffer:DMF:Triton X-100, v/v), Triton X-100, v/v, v/v), Triton X-100, v/v). The solvent has little effect on the spectrum of the native protein (Fig. 1, a, and b), but the addition of thiophenol slowly displaced the protein band near 400 nm to longer wavelengths until the time-invariant spectrum shown in Fig. 1c was obtained, typical of the [Fe₅S₄(SPh)₂]²⁻ anion (Kurtz, 1982). This required an incubation period of 80-100 min but the time could be decreased if the DMF concentration was increased. In a solvent mixture of 64:30:6 (buffer:DMF:Triton X-100, v/v), complete extrusion could be obtained in 20-25 min. Results from three such experiments gave values of 1.94 ± 0.07 for the mole ratio [4Fe-4S]/ferredoxin. The DMF/Triton X-100 procedure therefore is applicable to simple proteins containing [4Fe-4S] centers.

Extrusion of the iron-sulfur center of hydrogenase II by the DMF/Triton X-100 method was not successful. In the solvent mixtures 5-40% DMF (with 6% Triton X-100, v/v) or 5-8%...
Hydrogenase II of Clostridium pasteurianum

for hydrogenase II, the absorption coefficients at 275 and 420 nm were 86,900 and 23,000 M⁻¹ cm⁻¹, respectively (values from three preparations). Hydrogenase II under H₂ could be further reduced by sodium dithionite as indicated by the decrease in absorption in the 400-600-nm range (Fig. 4a). If the H₂ gas phase was replaced by Ar (no dithionite present) there was no change in the absorption spectrum but the admission of air to the sample increased the absorption in this region (Fig. 4b), a characteristic of iron-sulfur proteins. For the air-oxidized enzyme, the absorption coefficient at 420 nm was 27,200 M⁻¹ cm⁻¹, corresponding to 3400 M⁻¹ cm⁻¹/iron atom.

Chemical analysis showed that if hydrogenase II was treated with potassium ferricyanide, iron was released from the enzyme. When hydrogenase II (0.12 mM) was incubated under Ar with potassium ferricyanide (10 mM), the greenish solution was visibly bleached within 2 min. The absorption spectrum

![Absorption Spectra](image)

**FIG. 1.** Active site extrusion of ferredoxin in aqueous DMF/Triton X-100. The experiment was carried out as described under “Materials and Methods.” a, ferredoxin (28 μM) in 100 mM Tris/HCl, pH 8.0; b, solution in a diluted with DMF and Triton X-100 to give a final solvent composition of 79:14:7 (buffer:DMF/Triton X-100, v/v); c, solution in b after the addition of thiophenol (20 mM) recorded after 100 min.

Triton X-100 (with 14% DMF, v/v) with thiophenol (20-100 mM), the protein precipitated. In DMF/Triton mixtures, the protein was soluble, but the addition of thiophenol caused rapid precipitation. This extrusion medium therefore appears not to be suitable for this particular protein. We therefore turned to the previously described HMPA method (Gillum et al., 1977) using the same preparations of hydrogenase II. As shown in Fig. 2, the active centers of hydrogenase II were extruded in HMPA (80%, v/v). Upon addition of thiophenol, the spectrum was red-shifted (Fig. 2, a and b) and after 16 min a constant spectrum (Fig. 2c) was obtained. The latter unambiguously demonstrates the presence of [4Fe-4S] centers, rather than [2Fe-2S] centers or mixtures of centers in hydrogenase II. The peak at 458 nm is characteristic of [Fe₄S₄(SPh)₄]²⁻ and although the A₄58/A₅50 ratio is low (1.87 ± 0.09 from three determinations) compared to the pure cluster (A₄58/A₅50 > 2.02), this ratio is similar to that obtained for the extrusion of hydrogenase I (Gillum et al., 1977). From the A₄58 values, the mole ratio of [4Fe-4S]/protein was 2.04 ± 0.11 for hydrogenase II. These results therefore indicate that hydrogenase II contains 2 [4Fe-4S] clusters/mol in agreement with the measured metal and acid-labile sulfide content.

**Absorption Spectra of Hydrogenase II**—Hydrogenase II as isolated in the presence of sodium dithionite (1 mM) was dark green in color and could be easily distinguished from other proteins during column chromatography. Dithionite was removed by anaerobic gel filtration under H₂ and the UV-visible absorption spectrum of the hydrogenase under H₂ is given in Fig. 3a. This shows increased absorption from 700 to 275 nm with a broad band around 420 nm, typical of many other hydrogenases (see Adams et al., 1981). Assuming Mₕ = 53,000

![Absorption Spectra](image)

**FIG. 2.** Active site extrusion of hydrogenase II in aqueous HMPA. The experiment was carried out as described under “Materials and Methods.” a, hydrogenase II (12.4 μM) in HMPA (80%, v/v); b, solution in a recorded 1 min after the addition of thiophenol (50 mM); c, solution in b recorded after a further 15 min.

![Absorption Spectra](image)

**FIG. 3.** Absorption spectra of hydrogenase II. a, hydrogenase II (0.4 mg/ml) under H₂ in 50 mM Tris/HCl, pH 8.0, after desalting under H₂; b, hydrogenase II was treated with potassium ferricyanide as described in the text and after removal of the ferricyanide by gel filtration the spectrum of the isolated protein (0.35 mg/ml) was recorded.
Hydrogenase II of Clostridium pasteurianum

Fig. 4. Effect of redox state on the visible absorption of hydrogenase I and hydrogenase II. a, hydrogenase II (0.28 mg/ml) under H₂ in 50 mM Tris/HCl, pH 8.0, was obtained as described in Fig. 3a. Sodium dithionite (0.5 mM in 1.0 mM Tris/HCl, pH 8.0) was added to a final concentration of 2 mM. b, hydrogenase II (0.27 mg/ml) under H₂ was prepared as in a. The Ar-treated enzyme was obtained by 10 cycles of degassing and flushing with Ar. Hydrogenase II was then oxidized by degassing and exposing the sample to air. The upper and lower reduced curves were recorded 2 and 15 min after the gassing cycle with H₂, respectively. c, hydrogenase I (0.35 mg/ml) under H₂ (lower curve) was prepared by the same method used for hydrogenase II. Ar-oxidized hydrogenase I was then prepared as in b. The same sample was then re-reduced with H₂ by the same degassing/flushing procedure replacing Ar with H₂. The upper and lower reduced curves were recorded 2 and 15 min after the gassing cycle with H₂, respectively. d, H₂-reduced hydrogenase I (0.35 mg/ml) was prepared as in c. Sodium dithionite was then added as in a. The air-oxidized spectrum is reproduced from c for comparison.

For comparison, the absorption spectrum of hydrogenase I was examined under H₂, Ar, and air. The enzyme in H₂-saturated buffer gave an absorption curve similar to that of hydrogenase II (Fig. 4c). The absorption coefficient at 420 nm was 24,400 M⁻¹ cm⁻¹. However, if H₂ was replaced by Ar, hydrogenase I became more oxidized. Re-reduction with H₂ was a slow reaction taking 15 min before the H₂-reduced spectrum was obtained. Exposure of the sample to air gave a spectrum similar to that under Ar (Fig. 4c). The addition of sodium dithionite to the H₂-reduced enzyme resulted in a large decrease in the absorption around 420 nm (Fig. 4d). On the basis of the number of iron atoms in hydrogenase I and hydrogenase II, the decrease in absorbance from H₂-reduced to dithionite-reduced is 670 M⁻¹ cm⁻¹/iron (at 420 nm) for hydrogenase I, but only 240 M⁻¹ cm⁻¹/iron for hydrogenase II. If it is assumed that dithionite completely reduces both enzymes, hydrogenase II is reduced to a greater extent by H₂ than is hydrogenase I. The differences between the air-oxidized and H₂-reduced spectra are similar for hydrogenases I and II (640 and 520 M⁻¹ cm⁻¹/iron atom at 420 nm, respectively). This must be qualified since the activities of both enzymes are extremely sensitive to inactivation by O₂ (see below) and the air-oxidized state is not well defined.

Replacing H₂ with Ar did not affect the spectrum of H₂-reduced hydrogenase II (Fig. 4b) but H₂-reduced hydrogenase I became more oxidized (Fig. 4c). Hydrogenase I is extremely active in H₂ production and presumably equilibrates under Ar by producing H₂ and becomes more oxidized. Alternatively, the enzyme was oxidized by O₂ contamination during the degassing/gassing procedure. This would seem unlikely since hydrogenase II is as sensitive to O₂ inactivation as hydrogenase I (see below), yet it remained "reduced" under Ar. Although hydrogenase II is an extremely poor catalyst of H₂ evolution (see below), if the lack of oxidation of H₂-reduced hydrogenase II under Ar was purely a kinetic effect, one would expect its spectrum to eventually become more oxidized. However, this was not observed over a 15-min period and thus suggests that the enzyme is "trapped" in the H₂-reduced state and cannot become oxidized by reducing protons. Air-oxidized hydrogenase I was only slowly reduced by H₂ but the reason for this slow reaction is not obvious. The mechanistic implications of these data are discussed below.

EPR Spectra of Hydrogenase II—Hydrogenase II when prepared in dithionite-containing buffer (pH 8.0) gave a rhombic EPR spectrum at 16 K typical of a reduced ferredoxin-type or [4Fe-4S]⁴⁺(2e,1⁺) cluster. The spectrum is almost identical to that obtained with the reduced 4Fe-ferredoxin from Bacillus steareothermophilus (Mullinger et al., 1975). The spectrum of the reduced hydrogenase corresponded to gₑ = 1.96, also typical of the ferredoxin cluster. Potassium ferricyanide is often used to generate an oxidized enzyme species but because of the instability of hydrogenase II to this reagent, thionine was used to prepare oxidized hydrogenase II. The EPR spectrum is given in Fig. 5b. The sharp rhombic spec-

Fig. 5. EPR spectra of hydrogenase II. a, hydrogenase II (5.6 mg/ml) was prepared in 50 mM Tris/HCl, pH 8.0, containing sodium dithionite (1 mM). b, as in a except the sample was diluted 5-fold with anaerobic buffer without dithionite and thionine was added to a final concentration of 0.6 mM. The spectra were recorded at 16 K with gain settings of (a) 1.6 × 10⁸ and (b) 6 × 10⁸. The other instrument settings were: microwave power, 20 milliwatts; frequency, 9.25 GHz; modulation amplitude, 1.0 millitesla at 100 KHz.
trum with g values above 2 (gmax = 2.04) is characteristic of an oxidized Hipip type [4Fe-4S]2+/3+ cluster. This signal is very similar to that obtained with hydrogenase I (gmax = 2.05; Chen et al., 1976) and with the hydrogenases of Megalosphera vulgaris (van Dijk et al., 1980) and Desulfovibrio vulgaris (Grande et al., 1982). The EPR signals from the oxidized and reduced forms of hydrogenase II were not observed above 50 and 30 K, respectively.

Catalytic Properties of Hydrogenase II - In the routine assay for H2 oxidation using methylene blue (0.05 mM) as the electron acceptor, hydrogenase II had a specific activity of 3000 units/mg. In the H2 evolution assay with methyl viologen (1 mM) as the electron carrier, the activity was 5.9 μmol of H2 evolved/min/mg, giving an activity ratio (H2 uptake/H2 evolution) of about 500. In the same assay systems, the values for hydrogenase I were 4700 units/mg and 510 μmol of H2 evolved/min/mg for a ratio of about 9. On this basis, hydrogenase II is preferentially unidirectional in favor of H2 uptake. Apparent Km and Vmax values for methyl viologen and methylene blue in their respective assays were determined with both hydrogenases (Table I). Both enzymes have similar and very high activities in the H2 uptake assay but hydrogenase I is over 500-fold more active in H2 production, emphasizing the unidirectionality of hydrogenase II. Both enzymes also reduce the dithionite-reduced methyl viologen or ferredoxin at constant 1 mM volume of sample injected (usually less than 2 μl) was so small that nonenzymic dye reduction by dithionite was not observed. Other researchers have reported very long lag periods in the H2 uptake assay (e.g. Lalla-Maharajh et al., 1983) or a requirement for O2 scavengers in the assay medium (van Dijk et al., 1979; see also Adams et al., 1981).

A comparison of the apparent Km values for the different electron carriers (Table I) shows remarkable agreement between hydrogenases I and II. The exception is methyl viologen in the H2 evolution assay. Hydrogenase I has a similar affinity for both oxidized and reduced methyl viologen yet hydrogenase II has an 18-fold greater affinity for the semiquinone. This difference is even more evident from a double reciprocal plot of the kinetic data obtained for hydrogenase II and methyl viologen in the H2 uptake assay (Fig. 6). The plot was linear in the range 0.1–7.0 mM and this was used to compute the values in Table I. Above 7 mM, a second linear reciprocal plot was obtained which corresponded to an apparent Km of 67 mM (Vmax = 840 units/mg). Hydrogenase I did not exhibit the same kinetics in this H2 uptake assay but with M. elsdendii hydrogenase (van Dijk et al., 1979) an extremely high Michaelis-Menten constant (171 mM) was reported for methyl viologen. The Km value we determined for the methyl viologen semiquinone and hydrogenase I (Table I) is similar to the value (8.8 mM) reported by Erbes and Burris (1978) although the latter used a partially pure enzyme preparation.

Methyl viologen and ferredoxin have a synergistic effect on the H2 evolution activity of hydrogenase I (Chen and Mortenson, 1974). The rate of H2 production by hydrogenase I in the presence of both electron carriers is up to 5-fold greater than that observed with either as the sole electron donor. This was not the case with hydrogenase II. For example, the rate of H2 evolution in the presence of methyl viologen (10 mM) increased when ferredoxin (0.05–0.20 mM) was added as an additional electron donor but the observed rate was no more

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**Table I**

**Apparent Km and Vmax values in the H2 evolution and H2 uptake assays**

All values were determined from Lineweaver-Burk plots under standard assay conditions as described under “Materials and Methods.” Vmax values are expressed as micromoles of H2 evolved or consumed/min/mg of protein. The ratios are calculated on the basis of unity for the Vmax value for H2 evolution with methyl viologen as the electron carrier.

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<th>Electron carrier and assay</th>
<th>Methyl viologen, H2 evolution</th>
<th>Methylene blue, H2 evolution</th>
<th>Methyl viologen, H2 uptake</th>
<th>Methylene blue, H2 uptake</th>
<th>Ferredoxin, H2 evolution</th>
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*From Chen (1978).
than the sum of the rates obtained with each as the sole electron donor.

Effect of pH on Catalytic Activity—The rate of H₂ oxidation catalyzed by hydrogenase II with methylene blue as the electron acceptor showed pH optima at 7.0 and 10.5 (Fig. 7). In contrast, the rate of H₂ uptake with methyl viologen as the electron acceptor increased slowly with increasing pH below 9 but sharply increased to a relatively high uptake value of 11.4. For comparison, the effect of pH on hydrogenase I-catalyzed H₂ uptake with methyl viologen as the electron carrier was also determined (Fig. 7). This gave a similarly shaped curve to hydrogenase II but was slightly acid-shifted with an optimum at pH 9.8. This may indicate some mechanistic similarities between the two enzymes although the rates of H₂ oxidation obtained with hydrogenase I are about 10 times higher. The absorption coefficient of the methyl viologen semiquinone decreases below pH 7.0 (Erbes and Burris, 1978). The activities in Fig. 7 are uncorrected for this effect and are therefore slight overestimates of the rate of H₂ oxidation. However, the H₂ uptake assay of hydrogenase II at pH 8.0 measures minimal activity with both methyl viologen and methylene blue as the electron carriers (Fig. 7).

The oxidized forms of methyl viologen and methylene blue under H₂ do not affect the pH of the media; therefore, the pH of the buffer used in the H₂ uptake assay is the true pH of the assay medium (prior to H₂ catalysis). However, this is not the case with the H₂ evolution assay using dithionite-reduced methyl viologen as the electron donor to hydrogenase. The reduction of methyl viologen by dithionite can be represented by the following set of equations where MV²⁻ and MV* represent the oxidized and reduced forms of methyl viologen, respectively:

\[ \frac{1}{2}SO_2^- + MV^2- + H_2O \rightarrow MV^* + HS0_3^- + H^+ \]  
\[ HS0_3^- = SO_2^- + H^+ \]

Dithionite oxidation proceeds via the radical SO₂⁻ (Equation 1) with a subsequent one-electron transfer to yield bisulfite and the reduced dye (Equation 2). The ionization of bisulfite (Equation 3) has a pKₐ of 6.9 (see Mayhew, 1978). The reduction of methyl viologen by sodium dithionite will therefore increase the acidity of the media via Equations 2 and 3.

To investigate the effectiveness of the buffers to be used in resisting this pH change, the pH values of the reaction mixtures were determined by adding methyl viologen (to 1 or 10 mM final concentrations) to sodium dithionite (20 mM) in the buffer. The buffers used were as in Fig. 7, all at final concentrations of 50 mM. With an initial pH of 7 or less, the pH of the medium was unaffected by the reduction of methyl viologen (1 mM). Above pH 7.0, 1 mM methyl viologen caused a small decrease in pH but with 10 mM methyl viologen large pH changes were observed. For example, at initial pH values of 7.03, 8.08, 9.00, 10.40, and 11.40, the pH values after the addition of 1 mM (or 10 mM) methyl viologen were 7.02 (6.72), 7.93 (7.37), 8.20 (6.76), 9.97 (9.35), and 11.11 (10.60), respectively. Increasing the buffer concentration to 100 mM negated the pH change with 1 mM methyl viologen and this was used to determine the effect of pH on hydrogenase activity. However, large pH changes were still observed using 10 mM methyl viologen at pH 8 and above, even with the increased buffering capacity of the media. It should therefore be noted that the reduction of methyl viologen by dithionite can have a drastic effect on the pH of the assay medium. The change in pH predicted by the above equations is observed and, as expected, ΔpH is dependent on the concentration of reduced methyl viologen.

The effect of pH on hydrogenase-catalyzed H₂ evolution is shown in Fig. 8. The activity of hydrogenase I increased steadily with decreasing pH with an optimum at pH 6.3 but a very different curve was obtained with hydrogenase II. The latter showed two pH optima at 9.1 and 5.8. This suggests a different mechanism of H₂ evolution for the two enzymes although from Fig. 7 they have a similar pH dependency in H₂ oxidation. The midpoint potential (EM) of methyl viologen is independent of pH but the EM of sodium dithionite varies with pH (Mayhew, 1978). Below pH 7.0, the latter limits the extent of methyl viologen reduction, i.e., the substrate concentration decreases. Increasing the methyl viologen concentration to 10 mM resulted in an increase in the activity of hydrogenase II below pH 8.0 (Fig. 8). At pH 9.0 and 10.3 (measured pH values before enzyme addition), the activity was less than 20% above that measured with 1 mM methyl viologen at the same pH, which suggests that the increased activity below pH 7.0 reflects the increased concentration of reduced methyl viologen rather than a change in the Kₚ value.

![Fig. 7](image1.png)

**Fig. 7. Effect of pH on H₂ oxidation activity.** The H₂ uptake assay was performed as described under “Materials and Methods” using methylene blue (0.05 mM; ●) or methyl viologen (10 mM; ○) as the electron acceptor. The activities were determined with hydrogenase II (●, ○) and hydrogenase I (○). The buffers used were: citrate, pH 5.0-6.0; Mops, pH 6.2-7.0; Tris/HCl, pH 7.5-8.4; glycine/NaOH, pH 8.8-10.2; ε-aminocaproic acid, pH 10.3-11.4; and phosphate/NaOH, pH 11.5-12.0. All buffers were at a final concentration of 50 mM.

![Fig. 8](image2.png)

**Fig. 8. Effect of pH on H₂ evolution activity.** The H₂ evolution assay was performed as described under “Materials and Methods” using methyl viologen as the electron carrier. The final concentrations of methyl viologen were 1.0 (●, Δ) and 10 mM (○). The activities were determined with hydrogenase II (●, ○) and hydrogenase I (Δ). The buffers used were as in Fig. 7 except the final concentrations were 100 mM.
The $K_a$ value for the methyl viologen semiquinone with hydrogenase I was not significantly changed by pH (Erbes and Burris, 1978). As was the case with the $H_2$ uptake assay, the routine $H_2$ evolution assay at pH 8.0 measures the minimal hydrogenase activity of three orders of magnitude difference in proton concentration.

Effect of $O_2$—Hydrogenase I is extremely sensitive to inactivation by $O_2$. The time required for a 50% loss in $H_2$ uptake activity after exposing the enzyme to air is 2-5 min (Erbes and Burris, 1976; Chen, 1978). Hydrogenase II was similarly sensitive to $O_2$ inactivation. For the pure enzyme, a 50% loss of activity occurred in about 2 min after air was admitted to the sample (Fig. 9). Chen (1978) reported a half-time value for activity loss of 30 min for a partially purified preparation of hydrogenase II. Hydrogenase II was not protected from $O_2$ inactivation to any extent by methyl viologen (10 mM) or ferredoxin (0.5 mM). Ferredoxin has been reported to stabilize hydrogenase I against $O_2$ inactivation (Khan et al., 1981) although the enzyme preparation used was only about 3% pure. Routinely, hydrogenase II was stored as pellets in liquid $N_2$ and there was no loss of activity over a 4-month period. In buffer (pH 7-8) containing dithionite (1 mM) at 4 °C, hydrogenase II lost no significant amount of activity after a 1-week incubation period.

Effect of Temperature—To determine the effect of temperature on the rate of $H_2$ catalysis by hydrogenases I and II, methyl viologen was used as the electron carrier in both the $H_2$ uptake and $H_2$ evolution assays. The Arrhenius plot for hydrogenase I-catalyzed $H_2$ uptake is given in Fig. 10. With both enzymes in both assay systems, the plots were linear in the range 15-50 °C. Between 50 and 70 °C, the rates of reaction decreased (Fig. 10) and the Arrhenius plots were used to calculate the activation energy ($E_A$) values and the temperature optimum for that reaction. The values are given in Table II for both hydrogenases. The $E_A$ values were obtained using concentrations of methyl viologen between 1.0 and 10.0 mM in both assay systems. The small variation in the values suggests that the activation energy is not dependent on the electron carrier concentration. All assays were carried out in Mops buffer since this is resistant to significant changes in pH with temperature. Between 25 and 45 °C the measured ΔpH was 0.10. Over the same temperature change, the pH of 50 mM Tris/HCl buffer decreased form 8.0 to 7.4; thus, any $E_A$ values determined with this buffer, e.g. Colbeau and Vignais (1981), should take the ΔpH into account.

Hydrogenase II catalyzed $H_2$ production and $H_2$ oxidation at about 1% of the rates observed with hydrogenase I with the methyl viologen concentrations used (cf. Table I) and in both assay systems the $E_A$ values for hydrogenase II were almost twice those obtained for hydrogenase I. The lower activity of hydrogenase II may therefore be due in part to the much higher energy barrier to be overcome in carrying out these reactions. This is substantiated to a large extent by a comparison with other hydrogenases. The specific activities in the $H_2$ evolution assay (using reduced methyl viologen) for Thioscapsa hydrogenase (Gogotov et al., 1978), hydrogenase II,

![Fig. 10. Arrhenius plot of hydrogenase I-catalyzed $H_2$ uptake. The $H_2$ uptake activity of hydrogenase I was determined at various temperatures under the assay conditions described under "Materials and Methods" except that the electron carrier was methyl viologen (10 mM) and 50 mM Mops, pH 7.2, was used as the buffer. Hydrogenase I (0.11 fig) was added to initiate the reaction after the reaction mixture had been equilibrated at the desired temperature for 15 min.

<table>
<thead>
<tr>
<th>$H_2$ evolution</th>
<th>$H_2$ uptake</th>
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<tbody>
<tr>
<td>$E_A$</td>
<td>$I_{max}$</td>
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<tr>
<td>$kd/mol$</td>
<td>°C</td>
</tr>
<tr>
<td>Hydrogenase I</td>
<td>35.9-38.4</td>
</tr>
<tr>
<td>Hydrogenase II</td>
<td>48.3-50.0</td>
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</tbody>
</table>
Rhodospirillum hydrogenase (Adams and Hall, 1979a), Escherichia coli hydrogenase (Adams and Hall, 1979b), and hydrogenase I, are 5, 6, 26, 100, and 500 μmol of H₂ evolved/min/mg. The reported E₅ values are 66, 50, 57, 37, and 36 kJ/mol, respectively. A comparison with other hydrogenases is difficult because of the different assay conditions and electron carriers that were used but the E₅ values reported all lie in the range 26-70 kJ/mol (see Adams et al., 1981).

The Tₚ₀ values given in Table II are from the initial rates of H₂ catalysis. Hydrogenase II was slightly more thermostable than hydrogenase I but both are much more sensitive to high temperatures than, for example, the hydrogenases of the photosynthetic bacteria (Gogotov et al., 1978; Adams and Hall, 1979a). Thiocapsa hydrogenase has a reported temperature optimum of 70 °C for H₂ evolution over 10 min, showing that the decreased rates of H₂ production observed with hydrogenases I and II were not due to the instability of the assay system. The absorbance of the methyl viologen semiquinone does not significantly change with temperature (Mayhew, 1978); thus, the H₂ uptake assay is also satisfactory at elevated temperatures.

Inhibitors of Hydrogenase Activity—Of the hydrogenases so far tested, all have been reported to be inhibited by CO (see Adams et al., 1981) except the soluble hydrogenase of Alcaligenes eutrophus (Schneider et al., 1979). The inhibition by CO of hydrogenase I-catalyzed H₂ uptake has been reported (Erbes and Burris, 1978; Thauer et al., 1974; Averill and Orme-Johnson, 1978) but not the effect of CO on H₂ evolution. CO severely inhibited H₂ production by both hydrogenases I and II but as shown in Fig. 11, hydrogenase I was more sensitive to CO inhibition. The effect of CO was noncompetitive with respect to methyl viologen (0.5–15 mM) and for hydrogenase II a linear reciprocal plot was obtained up to a pCO of 1000 Pa (Fig. 11), indicating that one molecule of CO was bound per molecule of hydrogenase II. From the slope, the inhibitor constant (Kᵢ) was calculated to be 340 Pa. With hydrogenase I, a linear reciprocal plot was obtained only up to a pCO of 200 Pa and the Kᵢ value was 110 Pa (from broken line in Fig. 11, inset). The nonlinearity of the reciprocal plots with increasing CO concentrations suggests a secondary effect of CO. The reported Kᵢ values for CO with other hydrogenases in the same assay system are between 1200 and 4700 Pa (Adams and Hall, 1979a, 1979b; Yagi et al., 1976; Lalla-Maharajh et al., 1983; Llama et al., 1979) showing that both the clostridial enzymes, notably hydrogenase I, are extremely sensitive to CO inhibition of H₂ production.

All of the above references also show that CO inhibition of these hydrogenases is completely reversible: the removal of CO by flushing with an inert gas restored the original hydrogenase activity. This was not the case with the clostridial hydrogenases. Hydrogenases I and II were incubated with CO (pCO of 2000 and 1600 Pa, respectively) for 5 min and the CO was then removed by flushing with Ar for 5 min followed by five cycles of degassing and flushing with Ar. The two enzymes retained 0.9% (I) and 15% (II) of their activities compared to controls treated similarly in the absence of CO. However, at very low CO concentrations (giving 20–40% inhibition of activity; see Fig. 11), full activity was restored to hydrogenase I by flushing with Ar, but not to hydrogenase II. It was reported that CO inhibits the H₂-dependent reduction of methyl viologen catalyzed by hydrogenase I reversibly and that this is competitive with respect to H₂ (Erbes and Burris, 1978; Thauer et al., 1974); therefore, the above experiments were repeated but H₂ was used in place of Ar to remove the CO (H₂ was then removed with Ar for the assay). With high concentrations of CO (pCO = 1000 Pa), H₂ had no effect with either hydrogenase, i.e. H₂ did not relieve inhibition by CO. With low concentrations of CO (pCO = 150 Pa) and hydrogenase II, there was some relief of CO inhibition (20% more activity was recovered with H₂ than with Ar) but the original activity was not recovered. Therefore, both hydrogenases bind CO irreversibly at high CO concentrations but when pCO is less than the Kᵢ value, hydrogenase I is reversibly inhibited but CO still binds to hydrogenase II irreversibly. Flushing with H₂ does not completely relieve the binding of CO to hydrogenase II at low CO concentrations. At high CO concentrations, degassing and flushing with H₂ had no effect with either enzyme.

The effect of other potential inhibitors of hydrogenase II activity was also investigated. Generally, hydrogenases are inhibited by heavy metal salts (Hg²⁺, Cu²⁺) but metal complexing reagents, e.g. azide, cyanide, EDTA, and 2,2'-bipyridyl, usually have little effect (see Adams et al., 1981). Hydrogenase I was reported to be strongly inhibited by mersalyl (a mercury derivative) (Nakos and Mortenson, 1971) but not by azide and cyanide (Thauer et al., 1974) or p-phenanthroline (Chen and Mortenson, 1974). The effect of these reagents on the activity of hydrogenase II is shown in Table III. In contrast to hydrogenase I, p-phenanthroline and 2,2'-bipyridyl inhibited hydrogenase II although we have shown that these reagents do not remove iron from the enzyme under nondenaturating conditions. Azide and EDTA had little effect but copper II sulfate (100 μM) totally inhibited hydrogenase II. Cyanide (10 mM) also had some inhibitory effect.

High concentrations of buffer have been shown to inhibit the activity of several hydrogenases (see Adams et al., 1981) but hydrogenase II was stimulated by both Tris/HCl and phosphate buffers. The activity of hydrogenase II (both H₂ evolution and H₂ uptake) in 1.0 M Tris/HCl, pH 8.0, or 1.0 M potassium phosphate, pH 7.2, increased by about 60 and 160%, respectively, compared to the activities in these buffers at a concentration of 50 mM. The H₂ evolution activity of hydrogenase I decreased by about 15% in 1 M Tris/HCl, pH 8.0. The activity of M. elsdenii hydrogenase was also reported to be stimulated by high ionic strength (van Dijk et al., 1980).

Both hydrogenases I and II were susceptible to proteolytic digestion but the active sites were more sensitive to chymotrypsin digestion than to substrate digestion (Yagi et al., 1976).

**Fig. 11.** Inhibition of H₂ evolution activity by CO. The production of H₂ from reduced methyl viologen was determined with hydrogenase I (□) and hydrogenase II (●) as described under "Materials and Methods" except the Ar gas phase was replaced by CO/Ar mixtures. Hydrogenase I (0.45 μg/ml) or hydrogenase II (18 μg/ml) was equilibrated at 30 °C in the presence of CO at the indicated concentration for 5 min and methyl viologen (1 mM) was then added to start the reaction. The results are expressed as a percentage of the activity determined in the absence of CO, or by a reciprocal plot (inset) where V₀ and V are the H₂ evolution activities in the absence and in the presence of CO, respectively.
Hydrogenase II was assayed by H₂ uptake with methylene blue as the electron acceptor as described under "Materials and Methods" except the reaction mixture also contained the inhibitor (1.0 or 10 mM). All reactions were performed in 50 mM potassium phosphate buffer, pH 7.2, except for copper II sulfate where 50 mM Tris/HCl, pH 8.0, was used. Results are expressed as a percentage of the activity observed in the absence of inhibitor.

<table>
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<th>Inhibitor</th>
<th>Residual activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.0 mM</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>36</td>
</tr>
<tr>
<td>EDTA</td>
<td>108</td>
</tr>
<tr>
<td>2,2'-Bipyridyl</td>
<td>66</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl fluoride</td>
<td>61</td>
</tr>
<tr>
<td>Copper II sulfate</td>
<td>0</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>108</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>107</td>
</tr>
</tbody>
</table>

**TABLE III**

**Inhibition of hydrogenase II**

The separation during purification of more than one hydrogenase activity has been found with many organisms, e.g. *M. eledendii* (van Dijk et al., 1979), *Methanobacterium thermoautotrophicum* (Jacobson et al., 1982), *Anaeraba clyndrica* (Tel-Or et al., 1978), and *D. desulfuricans* (Kruger et al., 1982) but it is not clear if the different activities represent different enzymes or complexes of the same enzyme (see also Adams et al., 1981). It is only from the aerobic hydrogen bacterium *Alcogienes eutrophus* (Schneider et al., 1979; Schink and Schlegel, 1979) and from the methanogenic bacterium *Methanobacterium formicicum* (Jin et al., 1983) that two hydrogenases have been purified and characterized and have been shown unambiguously to be two distinct enzymes. Our data with *C. pasteurianum* shows that this organism also contains two very distinct hydrogenase species that differ in molecular structure, metal content, catalytic activity, and sensitivity to inhibitors.

Neither hydrogenase I nor hydrogenase II of *C. pasteurianum* contained metal atoms other than iron in significant amounts. Nickel has recently been implicated to be part of the active center of several hydrogenases. Isotopic substitution analyses and/or EPR studies have shown that nickel is part of the hydrogenases from *D. gigas* (LeGall et al., 1982; Cammack et al., 1982), *M. thermoautotrophicum* (Albracht et al., 1982a) *Vibrio succinogenes* (Unden et al., 1982), *A. eutrophus* (Friedrich et al., 1982), and from two strains of *D. desulfuricans* (Kruger et al., 1982; Lalla-Maharaj et al., 1983). Nickel is also required for the biosynthesis of hydrogenase in some other organisms (Takakuwa and Wall, 1981; Partridge and Yates, 1982; Albracht et al., 1982b). The two hydrogenases of *C. pasteurianum* are so far the only reported hydrogenases that do not contain nickel, showing that nickel is not obligatory for H₂ catalysis by hydrogenase. All the above enzymes function in vivo to primarily consume H₂, whereas hydrogenase I of *C. pasteurianum* has the well defined physiological role of H₂ production. The latter enzyme is also much more active in H₂ evolution in vitro than the other hydrogenases. One could speculate that nickel serves to modulate hydrogenase activity in favor of H₂ consumption and the data on the nickel content of hydrogenases from other H₂ producing organisms, e.g. *M. eledendii*, *E. coli*, are eagerly awaited. The physiological role of hydrogenase II is at present unknown.

Our analyses showed that hydrogenase II of *C. pasteurianum* contains 8 iron and 8 acid-labile sulfide g atoms/53,000 g of protein. Accepting the inherent error in these analyses, we often found values of slightly above eight in both cases although no iron could be removed from the enzyme by chelators under non-denaturing conditions. Analyses of iron-sulfur proteins for iron and acid-labile sulfide typically give values slightly less than the actual value and one "rounds up" to the nearest integer. However, extrusion studies with hydrogenase II indicated the presence of two [4Fe-4S] clusters in agreement with 8 iron atoms/molecule. We therefore feel that rather than hydrogenase II containing an additional iron center, either the molecular weight is slightly underestimated or the enzyme does not give a true value in the determination of protein.

The new medium for the extrusion of 2Fe clusters reported by Kurtz (1982) was modified to extrude [4Fe-4S] centers from *C. pasteurianum* ferredoxin but this method was not applicable to hydrogenase II. Analysis by the HMPA method unequivocally showed the presence of [4Fe-4S] centers in hydrogenase II. Several iron-sulfur proteins have recently been reported to contain 3Fe-clusters (see Beinert et al., 1983) but these extrude as [2Fe-2S] clusters (Kurtz et al., 1979); therefore hydrogenase II does not contain 2Fe or 3Fe centers. The EPR spectra of hydrogenase II can be nicely interpreted assuming the presence of two [4Fe-4S] clusters. The reduced enzyme gives a spectrum (g₉ < 2) typical of a reduced ferredoxin [4Fe-4S]^{2+} cluster which disappears upon oxidation to give a sharp rhombic signal with g₉ > 2, typical of a HipI or [4Fe-4S]^{2+} cluster. The lack of complexity in both spectra is indicative of each signal arising from a single noninteracting cluster (Fig. 5). The reduced enzyme is therefore equivalent to (C⁻, C⁺) and the oxidized form is (C⁻, C²⁻) (see Adams et al., 1981). The EPR spectra of hydrogenase II are very similar to those obtained from hydrogenase I (Chen et al., 1976) except that the latter has two spin-coupled reduced ferredoxin (C⁺) clusters which give rise to a complex EPR signal. The EPR signals with g₉ > 2 from the oxidized forms of the two clostridial hydrogenases are remarkably similar. However, an oxidized 3Fe cluster also exhibits an EPR signal with g > 2 (see Beinert et al., 1983 and references therein). This signal is isotropic and is therefore distinct from the strong rhombic signals observed with the clostridial hydrogenases. The absence of 3Fe cluster in these hydrogenases is also supported by the metal analyses and the extrusion data. The 4Fe cluster giving rise to the rhombic EPR signal has been proposed as the site of H₂ catalysis (Chen et al., 1976; Chen, 1978). A mechanism of catalysis involving the "superreduced" state of this cluster, i.e. (1+) state, has recently been proposed by Veeger and co-workers (Grande et al., 1982).

When hydrogenase II of *C. pasteurianum* was treated with ferricyanide, all of the iron atoms were lost from the enzyme. Treatment of *C. pasteurianum* ferredoxin with ferricyanide causes the conversion of 4Fe clusters to 3Fe clusters (Thompson et al., 1981) but hydrogenase II was particularly susceptible to this oxidant. Preliminary experiments have shown that, after exposure to ferricyanide for less than 1 min hydrogenase II exhibited an isotropic EPR signal at g > 2. The

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3 M. W. W. Adams, unpublished data.
Hydrogenase II of Clostridium pasteurianum

destruction of the enzyme by ferricyanide may therefore involve the intermediate formation of 3Fe centers. The effect of ferricyanide on hydrogenase I has not been investigated but the enzyme can be reversibly oxidized and reduced with ferricyanide and H₂ or dithionite (Chen et al., 1976).

The catalytic properties of hydrogenases I and II are very different but the term “uptake” hydrogenase is not fully descriptive of hydrogenase II. With methyl viologen as the electron carrier, hydrogenase II is a relatively inactive hydrogenase in catalyzing both H₂ evolution and H₂ uptake. Its activity is comparable to the very low values reported for the hydrogenases of the photosynthetic bacteria (Gogotov et al., 1978; Adams and Hall, 1979a; Llama et al., 1981). It is only in the H₂ uptake assay with methylene blue as the electron acceptor that hydrogenase II is extremely active, comparable to hydrogenase I. The apparent Kₐ values for the electron carriers tested are remarkably similar for the two enzymes, except for the methyl viologen semiquinone where there is a 30-fold difference. The ease with which hydrogenase II saturates with reduced methyl viologen may help explain the low activity. The hydrogenase from M. elsdenii is extremely active in H₂ production and is not easily saturated with methyl viologen (van Dijk et al., 1979). The pH dependence of H₂ evolution catalyzed by hydrogenase II is very unusual in that it is biphasic and optimal at pH 9.1. For all other hydrogenases so far examined, a typical bell-shaped curve is obtained with an optimum pH value of 7 or less (see Adams et al., 1981 and references therein).

The differences in pH dependency between the two clostridial hydrogenases presumably reflect differences in the mechanism of H₂ catalysis. This is further supported by the large discrepancies in the activities of the two enzymes, in their respective activation energies in both assay systems and in their sensitivities to inactivation by CO. Hydrogenase I was more sensitive to CO than hydrogenase II, and the rate of CO inhibition of hydrogenase I by CO. Hydrogenase II was not limited by the rate of H₂ catalysis, i.e., the cleavage or formation of H₂ (Equation 7), because of the high rates of H₂ oxidation observed with methylene blue as the electron acceptor. Hydrogenase I reduced methylene blue and methyl viologen with H₂ at high rates (methyl viologen was slightly less active; Table I); therefore, hydrogenase I may well be limited by the rate of covalent bond formation or breakage (Equation 7). That is, the removal of H₂ from the catalytic site (Equation 8) does not limit H₂ production by hydrogenase I and the redox potential of the electron acceptor (which influences Equations 4 and 6) does not dramatically affect the observed rates of H₂ oxidation. Hydrogenase I is thus an extremely active enzyme limited by Equation 7. Hydrogenase II is also an extremely active catalyst (viz. Equation 7) but is limited by Equation 8. High rates of H₂ catalysis by hydrogenase II are therefore only observed in H₂ oxidation using an electron acceptor with a much more positive potential than the H₂ electrode (Eₐ = -420 mV). Experiments to further probe the mechanistic differences between the two enzymes are currently in progress.

Hydrogenases I and II differ considerably from the point of view of H₂ catalysis as described above but structurally the similarities are striking. Hydrogenase II is of slightly lower molecular weight and lacks what appears to be one [4Fe-4S]²⁺⁻⁻ cluster compared to hydrogenase I. We have previously shown that the cellular concentration of hydrogenase II seems to increase with cell growth, whereas the content of hydrogenase I remains unchanged. A tempting speculation is therefore that hydrogenase II is a primary breakdown product of hydrogenase I which accumulates during cell growth. The loss of a single [4Fe-4S] center and a small stabilizing peptide from hydrogenase I might confer the described properties on hydrogenase II. We are currently performing peptide analysis and immunological studies to investigate this possibility.

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