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Structural and Catalytic Properties of Hydrogenase from *Chromatium*†

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ABSTRACT: The enzyme hydrogenase, from the photosynthetic bacterium *Chromatium*, was purified to homogeneity after solubilization of the particulate enzyme with deoxycholate. The purification procedure included ammonium sulfate fractionation, treatment with manganous phosphate gel, heating at 63°, DEAE-cellulose chromatography, and isoelectric focusing. The last step gave two active enzyme fractions with isoelectric points of 4.2 and 4.4. It was shown that the two fractions were different forms of the same protein. The enzyme was obtained in 23% yield and was purified 1700-fold. The enzyme had a molecular weight of 98,000, a sedimentation coefficient of 5.16 S and gave a single protein and activity band on disc gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis gave a single band of mol wt 50,000, suggesting that the active enzyme was composed of two subunits of the same molecular weight. The pure hydrogenase contained four atoms of iron and four atoms of acid-labile sulfide, and had a visible absorption peak at 410 nm. Electron paramagnetic resonance (EPR) spectroscopy at 10–15 K showed a free-radical signal at $g' = 2.003$ in the oxidized enzyme and signals at $g' = 2.2$ and $g' = 2.06$ in the reduced enzyme. These findings suggest that *Chromatium* hydrogenase is an iron-sulfur protein. The pure hydrogenase catalyzed the exchange reaction between H_2 and HDO or HTO, the reduction of Benzyl Viologen and Methylene Blue, and the evolution of hydrogen from reduced Methyl Viologen. The mechanism of hydrogen activation was shown to be heterolytic cleavage to an enzyme hydride and a proton. Hydrogenase could not catalyze reduction of pyridine nucleotides or ferredoxin with H_2. The effect of pH and various inhibitors on the enzymatic activity has been studied.

The enzyme hydrogenase catalyzes the reversible activation of molecular hydrogen and is unique in that its substrate, H_2, is the simplest molecule known. The activation of hydrogen can be demonstrated in a number of ways. From studies on the enzyme-catalyzed conversion of para hydrogen and the exchange between hydrogen and HDO (Krasna and Rittenberg, 1954) or HTO (Anand and Krasna, 1965), it was concluded that hydrogenase cleaves hydrogen heterolytically to form an enzyme hydride and a proton.

In cells containing hydrogenase, the enzyme can reduce various substrates with hydrogen or evolve hydrogen gas from substrates with low redox potentials. In photosynthetic organisms, hydrogen can serve as the reducing agent under anaerobic conditions and reduce pyridine nucleotides. In extracts of the photosynthetic bacterium, *Chromatium*, the reduction of NAD^+ by hydrogen requires ferredoxin. This was demonstrated by Weaver et al. (1965) who also reported that the enzymatic activity was present in the soluble portion of the extract. Buchanan and Bachofen (1968), while confirming the ferredoxin requirement, found only 25% of the H_2–NAD^+ reducing activity of the extract in the soluble portion with no activity in the particulate fraction. Recombination of the soluble and particulate fractions restored the original H_2–NAD^+ activity, yet both fractions contained equal amounts of hydrogenase activity as measured by reduction of Benzyl Viologen with hydrogen. Feigenblum and Krasna (1970) found all the hydrogenase and ferredoxin-dependent H_2–NAD^+ reducing activity in the particulate fraction of *Chromatium* extracts. The particulate hydrogenase was solubilized with Triton X-100 or deoxycholate and the reduction of NAD^+ by hydrogen was dependent on either ferredoxin or Benzyl Viologen.

In order to study the mechanism of hydrogen activation by hydrogenase, the hydrogenase of *Chromatium* has been purified to homogeneity and its structural and catalytic properties studied as described in this report.

Experimental Section

All reagents were obtained from commercial sources and were of the highest purity available. *Chromatium* cells were grown as described previously (Feigenblum and Krasna,

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1970) and stored as a frozen paste. Hydrogenase activity was assayed by the deuterium exchange (Krasna and Rittenberg, 1954) or tritium exchange methods (Anand and Krasna, 1965; Feigenblum and Krasna, 1970). When assaying highly purified hydrogenase preparations, where the protein concentration was very low, bovine serum albumin (4 mg) was added to each assay. In the deuterium exchange assay, 1 unit of activity is defined as the appearance of 0.05 atom % D per hr (D_2O concentration of 10%). In the tritium assay, 1 unit is defined as an exchange rate of 1 mV/hr as measured in an ionization chamber with a 10^{12} Ω resistor (the ^3H_2O contained 1 mCi/ml). One unit of exchange activity, as defined, is equal to the activation of 0.287 μmol of hydrogen/min.

Manometric assays for reduction of substrates by hydrogen or evolution of hydrogen from reduced substrates were carried out as described by Feigenblum and Krasna (1970). Oxygen was removed by the use of Feiser's solution (Feiser, 1924), which contains 15 g of sodium hydrosulfite and 2 g of sodium anthraquinone-β-sulfonate in 100 ml of 20% potassium hydroxide. The manometric activities are expressed as micromoles of hydrogen consumed or evolved per minute.

Analytical Methods. Protein concentration was determined by the method of Lowry et al. (1951). Polyacrylamide disc gel electrophoresis was carried out at pH 9.5 and the gels were analyzed for protein and hydrogenase activity (Ackrell et al., 1966; Kidman et al., 1968). Sodium dodecyl sulfate gel electrophoresis, as described by Shapiro et al. (1967) and Weber and Osborn (1969), was used for estimation of the molecular weight of protein subunits.

The molecular weight of purified hydrogenase was determined by sucrose density gradient centrifugation (Martin and Ames, 1961) and by analytical ultracentrifugation (Yphantis, 1964). For the gradient centrifugation, sucrose solutions were prepared in 0.05 *M* phosphate buffer (pH 7.0) containing 0.001 *M* mercaptoethanol. Gradients were run at 7° for 18 hr in an SW-39 rotor at 34,000 rpm. Fractions were collected and assayed for hydrogenase activity. Ovalbumin, bovine serum albumin, and aldolase were used as standards. $s_{20,w}$ values were determined by sedimentation velocity in a Spinco Model E centrifuge.

Amino acid analysis was carried out by hydrolyzing purified hydrogenase in vacuo with constant boiling HCl for 22 hr at 110°, drying the sample over NaOH, and analyzing with a Beckman automatic amino acid analyzer. The instrument was calibrated with a standard amino acid mixture. Half-cystine was determined on a sample oxidized with performic acid (Hirs, 1956; Moore, 1963). Tryptophan was estimated spectroscopically by the methods of Bencze and Schmid (1957) and Edelhoch (1967). Both methods gave similar results.

Total iron was determined by atomic absorption with a Varian AA5 spectrophotometer. Acid-labile sulfide was measured by the method of Brumby et al. (1965).

Electron spin resonance measurements were performed on a Varian E-9 EPR spectrometer at 10–15 K. We are indebted to Dr. P. Aisen of the Department of Biophysics of the Albert Einstein College of Medicine for these analyses. For electron paramagnetic resonance (EPR) and iron determinations, the pure enzyme was first dialyzed against 0.05 *M* Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) buffer (pH 7.1), containing 0.1 *M* KCl and 5×10^{-5} *M* EDTA and then against iron-free 0.05 *M* Hepes (pH 7.1), containing 0.1 *M* KCl.

Table I: Purification of *Chromatium* Hydrogenase.

Step	Total Act. (Units)[a]	Total Protein (mg)	Sp Act. (Units/mg of Protein)	% Act. Recovd
Extract	3170	19,500	0.16	100
$(NH_4)_2SO_4$ fraction	2020	1,860	1.08	64
$MnCl_2$–heat	2480	115	21.6	78
High-speed supernatant fraction	2130	80.1	26.6	67
DEAE fraction	1520	12.2	125	48
Isoelectric focusing				
(a) 4.2	228	0.87	262	7
(b) 4.4	512	1.79	286	16

[a] The values in the table are based on the exchange assay and the units are defined in the Experimental Section. One unit of exchange activity is equal to the activation of 0.287 μmol of hydrogen/min.

Purification of Chromatium Hydrogenase. The method used for the solubilization of particulate hydrogenase by sonication in deoxycholate is that described by Kondo et al. (1957) with the omission of trypsin digestion which was found to be unnecessary (Feigenblum and Krasna, 1970). Except where noted, all steps were performed between 0 and 4° under normal aerobic conditions. For assay, the enzyme was activated by addition of hydrosulfite (exchange assay) or by the presence of an oxygen absorber in a center well (manometric assay). There was no loss of activity on exposure of the enzyme to air at any step in the purification procedure.

The packed cells from 40 l. of media were suspended in 3 vol of 3% sodium deoxycholate, and portions of 50 ml were treated by sonication in a 10 kc Raytheon sonic oscillator for 25 min. The sonicated solution was centrifuged at 18,000*g* for 20 min and the supernatant fraction recovered (extract).

The extract was diluted with 3 vol of 0.0067 *M* phosphate buffer (pH 7.0) and the pH raised to 8.0 by addition of 1 *N* KOH. This solution was incubated at 37°, with shaking, for 45 min after which the pH was brought to 7.0 with 1 *N* HCl. Saturated ammonium sulfate was added to bring the solution to 20% saturation, the mixture was centrifuged at 17,000*g* for 20 min, and the precipitate discarded. The supernatant solution was then brought to 50% saturation and centrifuged at 9000*g* for 15 min, and the supernatant fraction discarded. The precipitate was dissolved in 700 ml of 0.05 *M* phosphate buffer (pH 7.2), containing 0.01 *M* mercaptoethanol, and the solution clarified by centrifugation at 40,000*g* for 20 min ($(NH_4)_2SO_4$ fraction).

To the clear $(NH_4)_2SO_4$ fraction, 35 ml of 0.5 *M* $MnCl_2$ was added; the solution was stirred for 15 min and centrifuged at 17,000*g* for 15 min. The resultant supernatant solution was then heated at 63° for 10 min and cooled to 0°, and the suspension centrifuged at 40,000*g* for 20 min to yield a clear supernatant fraction ($MnCl_2$–heat).

This solution was concentrated to 25 ml in an Amicon ultrafiltration cell with a PM-30 membrane and the concentrated solution centrifuged at 100,000*g* for 90 min (high-speed supernatant fraction). The high-speed supernatant solution was dialyzed against 0.05 *M* phosphate buffer (pH 7.0), containing 0.001 *M* mercaptoethanol and applied to a DEAE-cellulose (DE-52) column (1.9 × 22 cm) equilibrated with the same buffer. The column was eluted with a linear phosphate gradient (pH 7.0, containing 0.001 *M* mer-

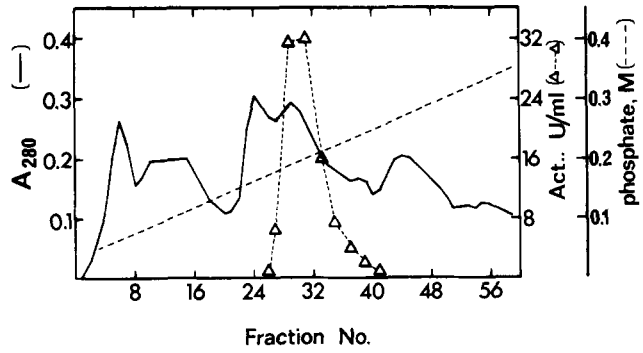

FIGURE 1: Elution pattern of hydrogenase from DEAE-cellulose column. Fractions of 8-ml volume were collected.

captoethanol) from 0.05 to 0.5 *M* at a flow rate of 25 ml/hr. Sixty 8-ml fractions were collected and assayed for exchange activity and absorbance at 280 nm. The active fractions were combined and concentrated to 4 ml in an Amicon ultrafiltration cell with a PM-10 membrane. This solution was dialyzed against 0.05 *M* phosphate buffer (pH 7.0) containing 0.001 *M* mercaptoethanol (DEAE fraction).

The DEAE fraction was then purified by isoelectric focusing between pH 3 and 5 using the apparatus and Ampholines supplied by LKB. The total concentration of Ampholines was 3%; 10 parts of pH 3–5 Ampholines were mixed with 1 part of pH 3–10 Ampholines. After the column reached equilibrium, fractions of 1-ml volume were collected and the activity found in two fractions at pH 4.2 and 4.4. These two fractions were centrifuged at 40,000*g* for 30 min and dialyzed against 0.05 *M* phosphate buffer (pH 7.0) containing 0.001 *M* mercaptoethanol to give two pure fractions, 4.2 and 4.4.

Results

Purification of Hydrogenase. A summary of the purification scheme developed for obtaining pure *Chromatium* hydrogenase is given in Table I. Extraction of the cells by sonication in deoxycholate followed by incubation at 37°, pH 8.0, solubilized the particulate hydrogenase. Most of the red pigments in the extract were removed by the ammonium sulfate treatment and $MnCl_2$ precipitation removed the remainder. Addition of $MnCl_2$ to the ammonium sulfate fraction in phosphate buffer produced a voluminous precipitate, probably manganous phosphate. This gel adsorbed other proteins and gave considerable purification. The hydrogenase activity at this stage was completely stable to heating at 70° for 10 min, and heating at 85° for 10 min inactivated only 40% of the activity. Heating at 63° for 10 min was, therefore, selected as a purification step. The elution pattern of the enzyme from DEAE-cellulose is shown in Figure 1; this step gave a five-fold purification. The final step, which gave pure hydrogenase, was isoelectric focusing; the results are presented in Figure 2A. The enzyme is separated into two distinct bands with p*I* values of 4.2 and 4.4. As can be seen from Table I, both forms of the enzyme have the same specific activity and, as will be shown below, are probably the same protein. This procedure gave a 1700-fold purification with 23% recovery of the initial activity.

To decide whether the 4.2 and 4.4 forms of the purified hydrogenase were different proteins or different ionic forms of the same protein, each separated form of the enzyme (from the peak tubes in Figure 2A) was subjected to isoelectric focusing a second time as shown in Figures 2B and 2C. It is clear that each form gave two components with p*I*

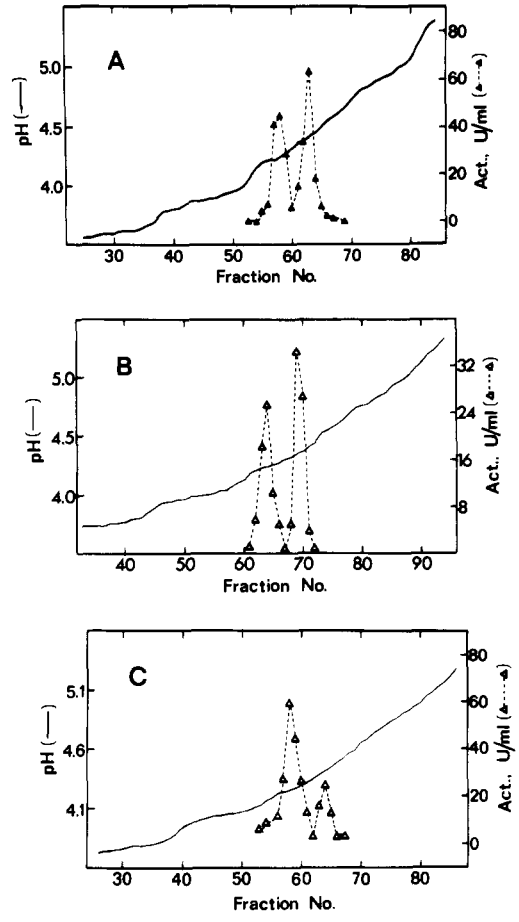

FIGURE 2: Profile of hydrogenase on isoelectric focusing. Ampholines were used in the following amounts: pH range 3–5, 5.0 ml; pH range 3–10, 0.4 ml. (A) Profile of enzyme after DEAE fractionation. Fractions containing 25 drops were collected. Fractions 57–59 were combined as the 4.2 form and fractions 61–64 were combined as the 4.4 form. The peak tubes from the two fractions separated in Figure 2A were subjected to a second isoelectric focusing and fractions of 16 drops were collected. (B) Profile of the 4.2 form of the enzyme. (C) Profile of the 4.4 form of the enzyme.

values of 4.2 and 4.4. This finding suggests that both peaks represent the same protein in different ionic forms. It is quite possible that the separation into two active forms is an artifact of the method perhaps due to complexing with Ampholines.

Molecular Weight. Sucrose density gradient centrifugation was used to estimate the molecular weight of the enzyme from the last steps in the purification. The enzyme after DEAE-cellulose chromatography had a molecular weight of 98,000 ± 8000. After isoelectric focusing the molecular weights were 100,000 ± 4000 for the 4.2 form and 98,000 ± 4000 for the 4.4 form.

To obtain more precise values for the molecular weight, the 4.4 form of the enzyme was analyzed by analytical ultracentrifugation. The $s_{20,w}$ value was 5.16 S. High-speed equilibrium sedimentation data (not shown) suggested a homogeneous preparation. Using a partial specific volume of 0.75 (calculated from the amino acid composition) and the equilibrium sedimentation data, the molecular weight was calculated to be 93,000 ± 4000.

Polyacrylamide gel electrophoresis of the 4.2 and 4.4 forms of the pure enzyme showed a single protein and activity band. Sodium dodecyl sulfate gel electrophoresis was used to estimate the subunit molecular weights. Both forms of the enzyme gave one major band and from the mobility

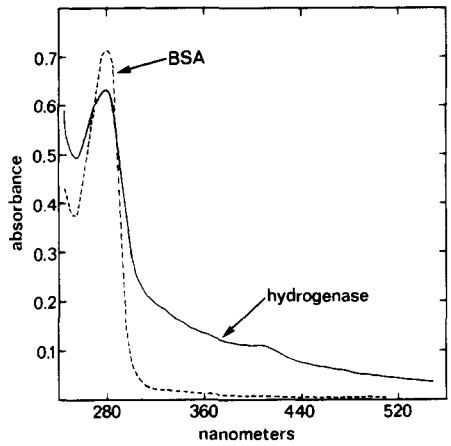

FIGURE 3: Absorption spectrum of *Chromatium* hydrogenase. The spectrum shown is that for both the 4.2 and 4.4 forms of the enzyme. The spectrum of bovine serum albumin is included for comparison.

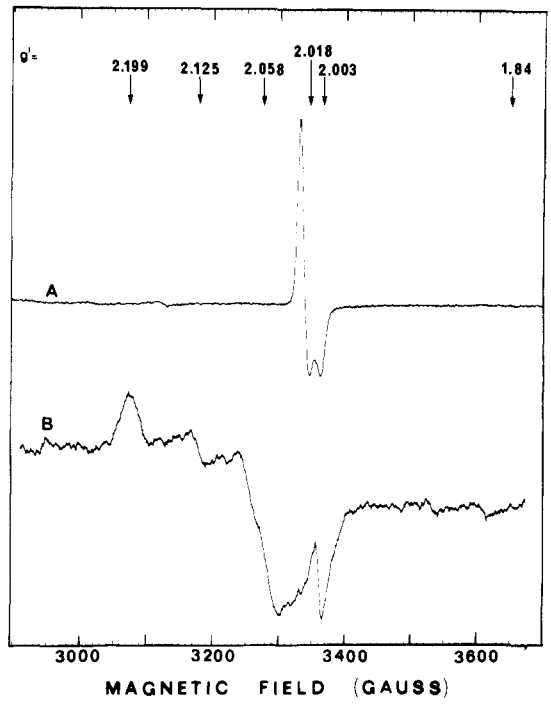

FIGURE 4: EPR spectrum of *Chromatium* hydrogenase. The 4.4 form of the enzyme at a concentration of 1.1×10^{-5} *M* in 0.05 *M* Hepes buffer (pH 7.1) containing 0.1 *M* KCl, was analyzed at 15 K: (A) oxidized enzyme as isolated; (B) after reduction with sodium dithionite; microwave power, 10 mW; modulation amplitude, 10 G; microwave frequency, 9.404 GHz; spectrometer gain, 1.25×10^3 (A) and 3.2×10^4 (B).

of this band (not shown), the subunit molecular weight was estimated to be 50,000 and 49,000 for the 4.2 and 4.4 enzymes, respectively. Considering that the molecular weight of the native enzyme was 93,000–100,000, it is reasonable to conclude that *Chromatium* hydrogenase consists of two subunits of molecular weight 50,000.

Chemical Properties of the Purified Enzyme. The absorption spectrum of hydrogenase in the ultraviolet and visible regions is shown in Figure 3. For comparison, the spectrum of bovine serum albumin is included. It is clear that hydrogenase absorbs in the visible with a peak at 410 nm. This absorbance is typical of iron–sulfur proteins (Orme-Johnson, 1973) and suggests that hydrogenase contains this functional group, probably at the active site. Both forms of the enzyme have identical absorption spectra and the millimolar extinction coefficients at 280 and 410 nm are 96 and 14, respectively. The extinction coefficient at 280 nm calculated from the aromatic amino acid content is 108. Addition of dithionite to the enzyme causes a slight decrease in the absorption at 410 nm.

Table II: Amino Acid Composition of Purified Hydrogenase.[a]

	No. of Residues	
	4.2 Form	4.4 Form
Lys	26	27
His	23	22
Arg	57	57
Asp	86	87
Thr	43	44
Ser	48	45
Glu	101	101
Pro	39	38
Gly	85	83
Ala	120	132
Half-Cys	14	12
Val	61	63
Met	14	15
Ile	48	50
Leu	76	76
Tyr	19	19
Phe	24	24
Trp	13	13

[a] The amino acid composition was calculated using 98,000 as the molecular weight of the enzyme. The details of the analysis are given in the Experimental Section. The values given are the average obtained from six separate determinations on three different enzyme preparations.

The amino acid analysis of the purified enzyme is given in Table II. Each value is the average of six separate determinations on three different enzyme preparations. In addition, the 4.4 form isolated by isoelectric focusing of the 4.2 form gave the same composition as the 4.4 form and vice versa. In general, both forms of the enzyme had the same amino acid composition which suggests that they are the same enzyme in different ionic forms. The only notable difference is in the value for alanine and it is slightly greater than the observed experimental error. Though the amide content was not determined, the high percentage of aspartic and glutamic acids is consistent with the low isoelectric point of the enzyme.

Analysis of the purified enzyme for iron and acid-labile sulfide showed 3.7 atoms of iron and 4.0 atoms of sulfide in the 4.2 form. The 4.4 form contained 3.6 atoms of iron and 3.9 atoms of sulfide.

The EPR spectrum of purified hydrogenase at 15 K is shown in Figure 4. In the oxidized state, there are two signals with axial symmetry at $g' = 2.003$ and $g' = 2.018$. On reduction, the 2.018 signal is lost and two new signals appear at $g' = 2.2$ and $g' = 2.06$ along with a very small signal at $g' = 2.125$. No signal is detected at $g' = 1.94$. The signals observed are consistent with hydrogenase being an iron–sulfur protein (Orme-Johnson, 1973).

Catalytic Properties of Purified Hydrogenase. Table III compares the activity of hydrogenase at different stages of purification with respect to reduction and oxidation of dyes. The enzyme, purified with respect to the exchange reaction, was able to catalyze the reduction of Methylene Blue and Benzyl Viologen with hydrogen and the evolution of hydrogen from reduced Methyl Viologen. It can be seen that as the enzyme is purified, the dye activity decreases compared

Table III: Reduction and Oxidation of Dyes by *Chromatium* Hydrogenase at Different Stages of Purification.[a]

	Exchange Act. (μmol of H_2/min per mg of Protein)	Ratio		
		Methylene Blue Reduction/ Exchange	Benzyl Viologen Reduction/ Exchange	Methyl Viologen Oxidation/ Exchange
Whole cells	0.029	8.4	2.5	0.86
Extract	0.046	5.5	3.0	0.89
$(NH_4)_2SO_4$ fraction	0.31	9.2	2.8	0.61
High-speed supernatant fraction	7.6	4.1	1.2	0.37
4.2 or 4.4 enzyme	82.1	3.9	0.95	0.43

[a] The manometric assays were performed as described in the Experimental Section. In each case, 10 μmol of substrate was added and the assays were done at two different enzyme concentrations to ascertain that the rates fell in the linear range. All activities are expressed as micromoles of H_2/minute per milligram of protein.

Table IV: NAD^+ Reduction by *Chromatium* Hydrogenase.[a]

	Rate of Exchange	Rate of NAD^+ Reduction in Presence of Cofactors			
		None	Benzyl Viologen	Clostridial Ferridoxin	Spinach Ferridoxin
Whole cells	0.67	0.005	0.039	0.012	0.016
Extract	2.24	0.011	0.017		
$(NH_4)_2SO_4$ fraction	0.94	0	0	0	
4.2 or 4.4 enzyme	2.24	0	0	0	

[a] NAD^+ (10 μmol) was present as substrate. Cofactors were added in the following quantities; Benzyl Viologen, 0.5 μmol; clostridial ferredoxin, 0.15 μmol; spinach ferredoxin, 0.025 μmol. The rates of exchange and NAD^+ reduction are expressed as micromoles of H_2/minute per milliliter of enzyme fraction.

Table V: Inhibition of Pure Hydrogenase.[a]

Compd Tested	% Inhibition	
	Exchange Reaction	Methylene Blue Reduction
Mercuric chloride	100	100
Silver nitrate	100	100
Copper sulfate	100	100
Sodium nitroprusside	44	88
2,3-Dimercapto-1-propanol	16	41
Potassium ferrocyanide	13	
Sodium mersalyl	9	6
Sodium iodoacetate	8	3
2,2'-Dipyridyl	8	
Sodium arsenite	7	
1,10-Phenathroline	7	
p-Hydroxymercuribenzoate		11

[a] The inhibitors were tested with the 4.4 form of the pure enzyme. All inhibitors were present at a final concentration of 10^{-3} *M*.

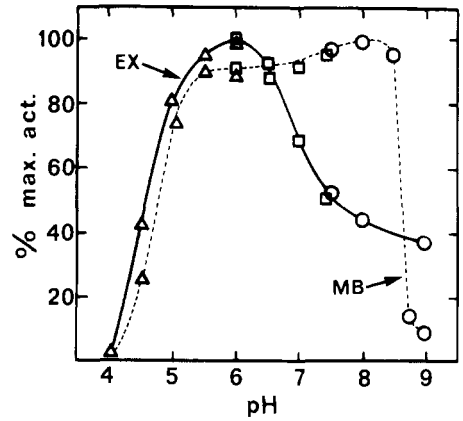

FIGURE 5: Effect of pH on the D_2O exchange and Methylene Blue reduction reactions, using purified *Chromatium* hydrogenase. Buffers were at a final concentration of 0.15 *M*: (△) citrate; (□) phosphate; (○) Tris.

to the exchange activity. This suggests that although the pure enzyme is capable of dye reduction and oxidation, there are factors present in the crude system which facilitate this reaction.

The activity of hydrogenase toward NAD^+ reduction at different stages of purification is shown in Table IV. In whole cells, NAD^+ reduction was stimulated by Benzyl Viologen or ferredoxin. The crude extract showed less stimulation by Benzyl Viologen, while after ammonium sulfate fractionation the enzyme could no longer reduce NAD^+ even in the presence of Benzyl Viologen or ferredoxin. It is clear that hydrogenase cannot reduce NAD^+ with hydrogen and that the reduction observed in crude systems is due to the presence of other enzymes or factors. The pure enzyme did not catalyze the reduction of ferredoxin with hydrogen.

Figure 5 compares the pH profile of the purified hydrogenase for the exchange reaction and the reduction of Methylene Blue. The exchange reaction shows a relatively sharp pH optimum at 6.0, while the Methylene Blue reduction shows a very broad pH profile from 5 to 8.5.

Activation of H_2 by hydrogenase was shown (Krasna and Rittenberg, 1954) to be a heterolytic process resulting in the formation of an enzyme hydride and a proton. The evidence for this mechanism is the demonstration of the formation of HD at a more rapid rate than DD when the exchange reaction is measured with H_2 in 100% D_2O. The ratio of HD to DD formation at all stages during the purification, from the whole cells to the pure enzyme, was 2.4–2.5. It is clear that the *Chromatium* hydrogenase catalyzes a heterolytic split of H_2 and that this mechanism is unaffected by the degree of purity of the enzyme.

Enzyme Inhibition. The effect of a number of inhibitors on the exchange and Methylene Blue reduction activity of pure hydrogenase is shown in Table V. The heavy metals, mercury, silver, and copper, completely inhibit the enzymatic activity. This was also found for the hydrogenase of *Scenedesmus* and *Proteus vulgaris* (Hartman and Krasna, 1964) and could be due to reaction with sulfhydryl, amino, carboxyl, or imidazole groups. Sodium nitroprusside, $Na_2[Fe(CN)_5NO]$, was shown to inhibit the hydrogenase of *Proteus vulgaris* (Krasna and Rittenberg, 1955) and

Desulfovibrio desulfuricans (Krasna et al., 1960) and, as shown in Table V, also inhibits *Chromatium* hydrogenase, although to a lesser extent. This inhibitor had a greater effect on Methylene Blue reduction. The other inhibitors tested had no effect on the enzymatic activity except for a partial inhibition of Methylene Blue reduction by 2,3-dimercapto-1-propanol. The failure of dipyridyl and phenanthroline to inhibit hydrogenase is in accord with our earlier observation (Hartman and Krasna, 1964) that chelating agents have no effect on active hydrogenase and suggests that the iron is firmly bound in the active enzyme.

The failure to observe inhibition by mersalyl, iodoacetate, *p*-hydroxymercuribenzoate, and arsenite suggests that modification of the available cysteine residues of *Chromatium* hydrogenase does not affect the catalytic activity, or that there are no available sulfhydryl groups. This is in contrast to other hydrogenases which are inhibited by these reagents (see Discussion).

Discussion

The procedure described above affords pure hydrogenase from the photosynthetic bacterium, *Chromatium,* in a reasonable yield. All the criteria applied (disc gel electrophoresis, sodium dodecyl sulfate gel electrophoresis, sedimentation analysis) indicate that the enzyme is homogeneous. The entire purification procedure and storage of the enzyme are done under aerobic conditions and no instability is ever observed. This contrasts with the pure hydrogenase from *Clostridium pasteurianum* (Nakos and Mortenson, 1971a) which is completely inactivated after exposure to air for 1 hr.

The last step in the purification, isoelectric focusing, separates the enzyme into two active fractions with p*I* values of 4.2 and 4.4. These two forms of the enzyme have the same molecular weight, subunit composition, behavior on gel electrophoresis, absorption spectra, amino acid analysis, EPR spectra, and iron and sulfide content. Therefore, these two forms of hydrogenase are probably the same enzyme in different ionic forms. The observation that each separated form gives rise to a mixture of the two on a second isoelectric focusing confirms this suggestion. However, it is not clear now whether they represent two different ionic forms or are artifacts of the separation due to complexing with the Ampholines. The isoelectric point of *Chromatium* hydrogenase is 4.2–4.4 while the hydrogenase from *Desulfovibrio vulgaris* has a p*I* of 6.25 (Yagi, 1970) and the enzyme from *Clostridium pasteurianum* has a p*I* of 5.0 (Nakos and Mortenson, 1971a). This fact suggests that these three hydrogenases, not surprisingly, are not identical.

The molecular weight of *Chromatium* hydrogenase is between 93,000 and 100,000 depending on which method is used for its estimation. Sodium dodecyl sulfate gels showed one major band of mol wt 50,000, indicating that the active enzyme is composed of two subunits of the same molecular weight. The hydrogenase of *Desulfovibrio vulgaris* has a mol wt of 60,000 and dissociates into two subunits of mol wt 30,000 (Yagi, 1970; Legall et al., 1971). Haschke and Capmbell (1971) reported that the molecular weight of this hydrogenase was 45,000 with a sedimentation constant of 2.58 S. The sedimentation constant for the *Chromatium* hydrogenase is 5.16 S. The hydrogenase of *Clostridium pasteurianum* has a mol wt of 60,000 (Nakos and Mortenson, 1971a,b; Chen and Mortenson, 1974). The report that it is composed of two subunits of mol wt 30,000 (Nakos and Mortenson, 1971a,b) has not been confirmed in the same laboratory (Chen and Mortenson, 1974) which has recently found that the enzyme does not dissociate into subunits.

Concerning the nature of the active site, the *Chromatium* hydrogenase contains four atoms of iron and four atoms of acid-labile sulfide per mole of active enzyme. The same content of iron and sulfide was reported for the hydrogenase of *Clostridium pasterianum* (Nakos and Mortenson, 1971a) and *Desulfovibrio vulgaris* (Legall et al., 1971). In contrast, Yagi (1970) found eight atoms of iron per mole of *Desulfovibrio* enzyme while Haschke and Campbell (1971) reported 1 atom of iron and 0.35 atom of sulfur per mole of this enzyme. Recently, the iron and sulfide content of the hydrogenase from *Clostridium pasteurianum* has been reported as 12 iron atoms and 12 acid-labile sulfide atoms (Chen and Mortenson, 1974).

The equivalence of iron and inorganic sulfide in *Chromatium* hydrogenase suggests that it is an iron–sulfur protein of the ferredoxin type. The visible spectrum of *Chromatium* hydrogenase, with a peak at 410 nm, supports this suggestion. This peak has also been observed in the other purified hydrogenases (Haschke and Campbell, 1971; Legall et al., 1971; Nakos and Mortenson, 1971a; Chen and Mortenson, 1974). The EPR spectrum is also consistent with an iron–sulfur chromophore which can be reduced (Orme-Johnson, 1973). In the reduced state, signals are observed at $g' = 2.2$ and $g' = 2.06$. There is no signal at $g' = 1.94$, though this signal was observed in other purified hydrogenases. The signals at $g' = 2.018$ and $g' = 2.003$ in the oxidized enzyme may be due to trace radical contamination, oxygen bound to the oxidized enzyme, or to the iron–sulfur cluster. A weak EPR signal at $g' = 2.015$ has been observed (Sweeney et al., 1974) in oxidized *Clostridial* type ferredoxins and attributed to a super-oxidized iron–sulfur cluster.

Chromatium hydrogenase was not inhibited by arsenite, mersalyl, iodoacetate, or *p*-hydroxymercuribenzoate. The failure of arsenite to inhibit is in accord with our earlier finding (Hartman and Krasna, 1964), whereas iodoacetate and *p*-hydroxymercuribenzoate caused complete inhibition of the hydrogenase from *Scenedesmus, Proteus vulgaris* (Hartman and Krasna, 1964), *Desulfovibrio desulfuricans* (Sadana and Rittenberg, 1963), and *Desulfovibrio vulgaris* (Haschke and Campbell, 1971). Though mersalyl inhibited the *Clostridium* hydrogenase (Nakos and Mortenson, 1971b), it was without effect on *Chromatium* hydrogenase.

The inhibition of hydrogenase by nitroprusside, $Na_2[Fe(CN)_5NO]$, has been confirmed in this laboratory on all hydrogenases studied and is observed with other iron pentacyano compounds, but not with hexacyano compounds (Krasna and Rittenberg, 1955). The inhibition may arise from the substitution of the sixth group (NO in this case) of the pentacyano compound by an active group of the enzyme.

Chromatium hydrogenase in whole cells or crude extracts reduces NAD^+ with H_2 in the presence of ferredoxin. This natural carrier can be replaced by Benzyl Viologen. Purified hydrogenase does not reduce NAD^+ even in the presence of carriers, nor does it reduce ferredoxin. The data in Table IV show that the ability to reduce NAD^+ is lost in the early stages of purification, probably due to the loss of unidentified factors or enzymes. A flavoprotein ferredoxin-NAD^+ reductase may be required, by analogy with ferredoxin-$NADP^+$ reductase isolated from spinach chloroplasts (Shin and Arnon, 1965; Avron and Jagendorf, 1957). This latter enzyme was required for the reduction of $NADP^+$ in a system containing ferredoxin and bacterial hydrogenase.

It should be noted that Benzyl Viologen has a much greater stimulatory effect on NAD^+ reduction in whole cells compared with the extract (Table IV) and may suggest that a factor or enzyme is required for activity with Benzyl Viologen. Nagi et al. (1968) have shown a specific NAD^+-Benzyl Viologen reductase in *Azobacter vinelandii* which catalyzes the reversible transfer of electrons between NAD^+ and Benzyl Viologen independent of ferredoxin and hydrogenase.

The inability of hydrogenase to reduce ferredoxin or NAD^+ is not surprising since hydrogenase is an enzyme which cleaves H_2 reversibly. The ability to transfer the activated hydrogen to a physiological acceptor probably depends on the presence of factors or enzymes specific for the acceptor. Therefore, in studying hydrogenase per se, it is essential to use an assay which directly measures the activation of H_2. The exchange reaction between hydrogen gas and HDO or HTO is such an assay and was used in this work. The results in Table III show that the reduction of Methylene Blue or Benzyl Viologen with hydrogen and the evolution of hydrogen from reduced Methyl Viologen are also inherent properties of the enzyme. The activity with these dyes is somewhat less with the pure enzyme than with cruder fractions suggesting that the crude preparations contain factors, enzymes, or electron carriers that facilitate the reaction with dyes. The different ratios of reduction to exchange for various substrates are related to the specific rate constants for each reaction and have been discussed elsewhere (Krasna and Rittenberg, 1956).

The HD/DD ratio of 2.5 in the exchange between H_2 and 100% D_2O shows that *Chromatium* hydrogenase catalyzes a heterolytic cleavage of hydrogen. A homolytic split, as in the case of platinum catalysis (Krasna, 1961), results in the predominant production of DD and not HD. In *Proteus vulgaris* this ratio was 5.0 (Tamiya and Miller, 1963) and in *Desulfovibrio desulfuricans*, 0.9 (Krasna et al., 1960). The lower HD/DD ratio in some organisms probably reflects the degree to which the enzyme hydride exchanges with the hydrogen of water.

The data presented in this investigation show that *Chromatium* hydrogenase contains four iron atoms and four acid-labile sulfides arranged as iron–sulfur complexes in the active site. The protein consists of two subunits of mol wt 50,000. We do not know how the subunits and iron–sulfur centers are arranged in the catalytically active enzyme or whether the two subunits are identical. There are a number of ways that the iron and sulfur atoms could be arranged on the two subunits. Each subunit could contain a binuclear iron–sulfur center with both subunits functional and each accepting a single electron. Though this configuration, with two single electron transfers, may not appear suited to a hydride mechanism, the two iron centers on the subunits may be close enough to each other in the active enzyme to function as a hydride acceptor. Another possible configuration would have a tetranuclear iron–sulfur center on one subunit which could accept two electrons and no metal site on the second subunit. This second subunit could be nonfunctional, have a regulatory role, or participate in the catalytic mechanism by binding the proton formed on splitting hydrogen or binding the other substrate. A third possible configuration would have a tetranuclear iron–sulfur center held by cysteine–iron bridges to both subunits. This would accommodate a hydride mechanism and ascribe an essential role to both subunits.

The resolution of these questions of the quaternary structure of hydrogenase must await more detailed structural studies now in progress.

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The Interaction of an Epoxide with Yeast Alcohol Dehydrogenase: Evidence for Binding and the Modification of Two Active Site Cysteines by Styrene Oxide†

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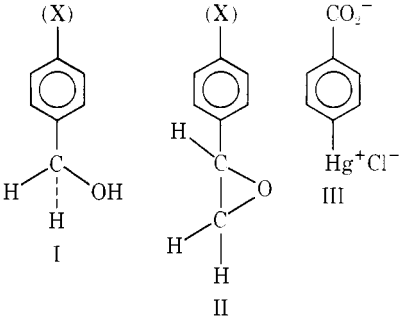
ABSTRACT: Yeast alcohol dehydrogenase is inactivated and alkylated by styrene oxide in a single exponential kinetic process. The concentration dependence of half-times for inactivation indicates the formation of an enzyme inhibitor complex, $K_I = 2.5 \times 10^{-2}$ *M* at pH 8.0. Reduced nicotinamide adenine dinucleotide (NADH), at a concentration of 3×10^{-4} *M* where $K_d \simeq 1 \times 10^{-5}$ *M*, has a small effect on kinetic parameters for inactivation. Although benzyl alcohol and acetamide-NADH increase the K_I for styrene oxide in a manner consistent with their dissociation constants, substrate also increases the rate of inactivation at high styrene oxide concentrations. The reciprocal of half-times for inactivation, extrapolated to infinite styrene oxide concentration, increases with pH between 7.6 and 9.0, p$K \simeq 8.5$. The stoichiometry of alkylation by [^{3}H]styrene oxide is 2.2 mol of reagent incorporated/mol of subunit, and is accompanied by the loss of 1.9 mol of sulfhydryl/mol of subunit; prior alkylation with iodoacetamide reduces the stoichiometry to 0.88:1, and increases the rate of labeling. Tryptic digests of enzyme modified with [^{14}C]iodoacetamide or [^{3}H]styrene oxide produce two major peptides which cochromatograph, indicating that styrene oxide and iodoacetamide modify the same cysteine residues. Previous investigators have reported that iodoacetate, iodoacetamide, and butyl isocyanate alkylate either of two reactive cysteines of yeast alcohol dehydrogenase; both cysteines cannot be modified simultaneously [Belke et al. (1974), *Biochemistry 13,* 3418]. The inactivation of enzyme by *p*-chloromercuribenzoate (PCMB) is reported here to be accompanied by the incorporation of 2.3 mol of PCMB/mol of enzyme subunits, in analogy with styrene oxide; the planarity of the alkylating agent appears to be an important factor in determining the stoichiometry of labeling.

The modification of enzymes by chemically reactive substrate analogs can provide insight into the nature of the active site side chains involved in catalysis. Inactivation of yeast alcohol dehydrogenase by iodoacetate and iodoacetamide (Whitehead and Rabin, 1964; Harris, 1964), and butyl isocyanate (Twu and Wold, 1973; Twu et al., 1973) has been studied by previous investigators in some detail. These three reagents are characterized by the following common properties: (i) the kinetics of the inactivation process do not indicate the reversible formation of enzyme–inhibitor complexes prior to inactivation; (ii) the coenzymes, NADH and NAD, protect against inactivation at concentrations consistent with their dissociation constants; (iii) inactivation is accompanied by the incorporation of 1 mol of reagent/mol of enzyme subunit; (iv) inactivation results in the alkylation of either of two reactive cysteines, depending on the reagent and the conditions of inactivation; and (v) both cysteines cannot be alkylated simultaneously, presumably due to overlap in sites.

In an effort to affinity label yeast alcohol dehydrogenase at the substrate binding site, and thereby obtain information concerning the side chain, pK = 8.25, which has been implicated in acid–base catalysis of the hydride transfer step (Klinman, 1975), a study of the inactivation of yeast alcohol dehydrogenase by styrene oxide, an analog of the aromatic substrate benzyl alcohol (Scheme I), was undertaken. Preliminary experiments indicated that the inactivation of yeast alcohol dehydrogenase by styrene oxide was characterized by saturation kinetics, suggesting the formation of a reversible enzyme–inhibitor complex, and that

Scheme I: Structural Similarity of Benzyl Alcohol (I), Styrene Oxide (II), and *p*-Chloromercuribenzoate (III).



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