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Structural Properties of Hydrogenase from *Clostridium pasteurianum* W5*

George Nakos† and Leonard E. Mortenson‡

ABSTRACT: Hydrogenase from *Clostridium pasteurianum* has an adsorption maximum at 280 mμ and a broad absorption between 350 and 500 mμ with a molar extinction coefficient at 400 mμ of 8000. Hydrogenase as isolated exhibits an intense, temperature-sensitive electron spin resonance signal of the $g = 1.94$ type. The signal is lost on oxidation and can be restored almost completely by incubation under H_2 or addition of dithionite in the absence of ferredoxin. Sodium dodecyl sulfate treated hydrogenase dissociates into two subunits of identical size with a molecular weight of about 30,000. The behavior of the enzyme on polyacrylamide gels made 8.0 M in urea suggests that its two subunits also have the same isoelectric point. Hydrogenase incubated with 4.0 M urea retains its activity for several hours. When dialyzed against 4.0 M urea, it rapidly loses iron, "acid-labile" sulfide, and enzymatic activity. Amino acid analysis of hydrogenase shows the presence of four half-cystine residues and the absence of tryptophan. All the other usual amino acids are present. Titration of hydrogenase with two mercurials and 5,5′-dithiobis(2-nitrobenzoic acid) indicates the presence of 12 SH equiv. Amino acid analysis, titration with mercurials, and elemental analysis establish the presence of both cysteine sulfur and acid-labile sulfide in hydrogenase. Treatment of hydrogenase with *o*-phenanthroline removes two of its four iron atoms but its hydrogen evolution and hydrogen-uptake activities are not decreased. Sodium mersalyl treatment removes the iron and acid-labile sulfide from the protein with concomitant loss of its enzymatic activity. The finding of hydrogenase "iso enzymes" in crude or partially purified preparations of the enzyme appears to be an artifact.

Hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) is involved in hydrogen metabolism and electron transport of a number of microorganisms (Gray and Gest, 1965), of certain plants (Renwick *et al.*, 1964), and probably of animals (Kuruta, 1962).

Hydrogenase from *Clostridium pasteurianum* has been purified to a state that shows one protein band and one coincident activity peak on analytical polyacrylamide gel electrophoresis (Nakos and Mortenson, 1971). These authors also found that hydrogenase from the same organism is an iron–sulfur protein containing 4.0 iron atoms and 4.0 "acid-labile" sulfide groups per molecule.

This paper describes some physical and chemical characteristics of the purified hydrogenase from *C. pasteurianum*. The theory that hydrogenase exists as iso enzymes (Ackrell *et al.*, 1966; Kidman *et al.*, 1969; Kleiner and Burris, 1970) is also investigated.

Materials and Methods

Chemicals. The chemicals used were obtained: from Fisher Scientific Co., *o*-phenathroline; from K & K Laboratories, disodium 1,2-dihydroxybenzene-3,5-disulfonate (Tiron); from Mann Research Laboratories, sodium *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate (sodium mersalyl), methyl viologen, and Ultra Pure urea; from Pierce Chemical

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Co., 5,5-dithiobis(2-dinitrobenzoic acid) (DTNB);[1] from Sigma Chemical Co., sodium dodecyl sulfate, *p*-hydroxymercuribenzoate (PMB), and dimethyl sulfoxide. All other reagents were commercially obtained and of the best available grade.

Growth of the Organism and Preparation of Crude Extracts. Cells of *C. pasteurianum* were grown using N_2 as the sole nitrogen source and cell-free extracts were prepared from dried cells by the method of Mortenson (1964).

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of Hydrogenase. The oxidation by hydrogenase of dithionite-reduced methyl viologen was monitored manometrically by measuring the volume of hydrogen gas evolved (Peck and Gest, 1956). Hydrogenase activity was also monitored by measuring reduction of methylene blue with H_2 as the reductant.

Preparation of Hydrogenase. The enzyme was purified according to the procedure of Nakos and Mortenson (1971).

Spectral Studies. The absorption spectrum of hydrogenase was measured with a Cary 14 recording spectrophotometer. The electron spin resonance spectrum was recorded with a Varian V-4502 spectrometer.

Sodium Dodecyl Sulfate–Polyacrylamide Disc Gel Electrophoresis. In the subunit studies the method of Shapiro *et al.* (1967) as described by Weber and Osborn (1969) was employed. Carboxymethylation was performed according to Sela *et al.* (1959).

Amino Acid Analysis. Samples of hydrogenase were desalted by dialysis, under H_2, against 4.0 l. of deionized air-free water for 4–6 hr. Hydrolyses were carried out in 6.0 N HCl at 110° for 24 and 48 hr. The hydrolysates were evaporated to dryness under vacuum over solid NaOH at 37° for 25 hr. The residues were analyzed by the method of Spackman *et al.* (1958) using a Technicon amino acid analyzer. The moles of each amino acid in an unknown sample is calculated on the basis of known amino acid standards run before and after the unknowns. The moles of protein corresponding to the total amino acids found for a particular sample was calculated by multiplying the moles of each amino acid by its corresponding residue weight, adding up the products, and dividing the total by the molecular weight of the protein. The number of residues of each amino acid per protein molecule was then derived by dividing the moles of each amino acid by the moles of protein. Performic acid oxidized samples were prepared according to Hirs (1956) and Moore (1963). Dimethyl sulfoxide oxidation was carried out as described by Spencer and Wold (1969). Tryptophan was measured spectrophotometrically (Bencze and Schmid, 1957), colorimetrically (Spies and Chamber, 1949), and by the acid–nihydrin method of Gaitonde and Dovey (1970). The partial specific volume ($\bar{v}$) of the apo protein (iron and acid-labile sulfide-free hydrogenase) was determined by the method of McMeekin *et al.* (1949).

Sugar Determinations. Total sugars were determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956) and the anthrone reagent (Scott and Melvin, 1953). Reducing sugars were measured according to Nelson (1954). The orcinol method was used for pentose sugar estimation (Mejbaum, 1939). Deoxy sugars were determined by the diphenylamine method (Dische, 1953).

Iron and Acid-Labile Sulfides. Total iron was determined by an *o*-phenanthroline method (Lovenberg *et al.*, 1963). Acid-labile sulfide was determined by a modification of the method of Fogo and Popowsky (1949) as described by Brumby *et al.* (1965).

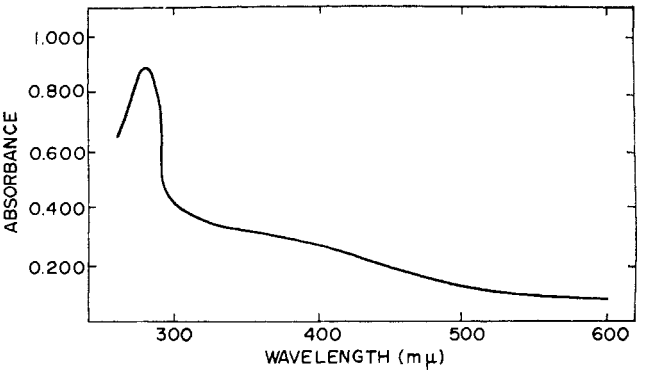

FIGURE 1: The absorption spectrum of hydrogenase under anaerobic conditions. Protein concentration based on Lowry *et al.* (1951) is 2.2 mg/ml. The solvent is 0.05 M Tris-HCl (pH 8). The spectrum was taken in a Cary 14 recording spectrophotometer.

Reaction with Sulfhydryl Reagents. The combined sulfide and sulfhydryl content was determined by the method of Ellman (1959) and by titration with sodium mersalyl (Klotz and Carver, 1961) and with PMB (Boyer, 1954).

Twenty microliters of 0.4% DTNB solution was added to 2.0 ml of air-free 0.05 M Tris-HCl (pH 8.0) buffer, containing 25 nmoles of hydrogenase. After 2.0 min the absorbance at 412 mμ was measured, and the sulfhydryl content was calculated using a molar extinction coefficient of 13,600 (Ellman, 1959).

For the anaerobic titration of hydrogenase with sodium mersalyl, 10-μl amounts of the mercurial solution (7 μmoles/ml) were added to a cuvet containing 3.5 mg of the enzyme in a total volume of 2.0 ml of air-free 0.05 M Tris-HCl pH 8 buffer. The decrease in absorbance at 400 mμ was recorded after 10–15 min and additional 10-μl amounts were added until no further decrease in absorbance occurred. Absorbance values were corrected for dilution.

Iso Enzymes. The method of Ackrell *et al.* (1966), employing the electrophoresis method of Davis (1964), was used to investigate the presence of hydrogenase iso enzymes. Gels (5%) containing 8.0 M urea were prepared according to Brewer and Ashworth (1969).

Results

Absorption Spectrum. The spectrum of hydrogenase (as isolated in the absence of air) in the 250- to 600-mμ region is shown in Figure 1. Assuming a molecular weight of 60,000 the millimolar extinction coefficients ($1 \times cm^{-1} \times mmole^{-1}$) at 280 and 400 mμ are 24.5 and 8.2, respectively. When the enzyme is exposed to air (O_2) for 0.5 hr no change in the absorption at 400 mμ is observed. Addition of 100-fold molar excess dithionite to the isolated enzyme reduces its absorption at 400 mμ about 10%. When the native enzyme is treated with a 20-fold molar excess sodium mersalyl it loses about 70% of its absorption at 400 mμ.

Electron Spin Resonance Spectrum. Figure 2 shows the electron spin resonance spectrum of hydrogenase as isolated. This is a preliminary study conducted in collaboration with G. Palmer, University of Michigan, Institute of Science and Technical Biophysical Research Division, Ann Arbor, Michigan. This signal disappears when the enzyme is exposed to

[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-hydroxymercuribenzoate.

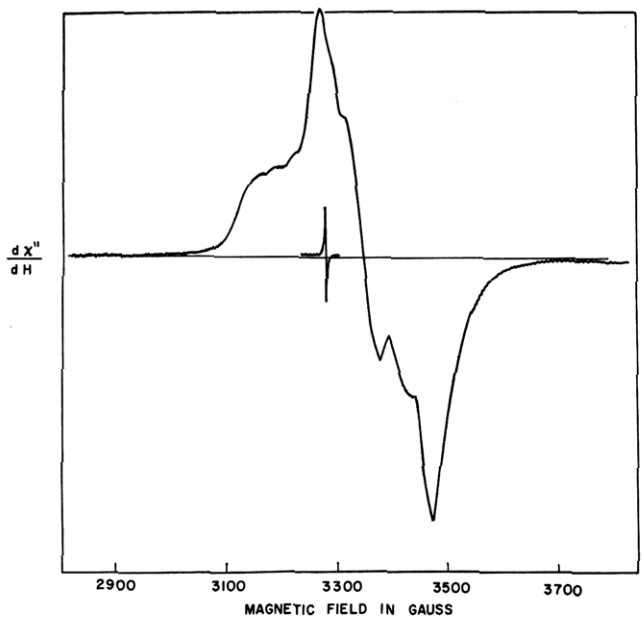

FIGURE 2: Electron paramagnetic resonance spectrum of 1 mM hydrogenase as isolated. The spectrum was obtained at 25°K in a Varian V-4500 electron paramagnetic resonance spectrometer operating at 9.197 GHz. Field modulation frequency = 100 kHz, amplitude ≈ 6 G. Power = 3 mW. Scan rate, 400 G/min. Time constant 0.3 sec. A free-radical field marker (g = 2.003) is also recorded. Data of G. Palmer.

air and it is almost restored upon reduction of the enzyme with dithionite or incubation under H_2. Further data and possible interpretation of the spectra will be published separately.

Subunit Structure. Treatment of the enzyme with 1.0% sodium dodecyl sulfate and 1.0% 2-mercaptoethanol for 5–10 hr and dialysis against 0.1% sodium dodecyl sulfate and 2-mercaptoethanol for 12–24 hr gives two protein bands on electrophoresis in the sodium dodecyl sulfate–polyacrylamide system (Weber and Osborn, 1969). The major protein band has a molecular weight of 30,000 and the other a molecular weight of 60,000 (Figure 3). When in the above treatment carboxymethylation (Sela *et al.*, 1959) is performed before dialysis, only one protein band with a molecular weight of 30,000 appears after electrophoresis in the same system (Figure 4, left). This indicates that hydrogenase is made up of two subunits of identical size each with a molecular weight of 30,000. The protein band with the molecular weight of 60,000 corresponds to the native enzyme the molecular weight of which, estimated by gel filtration, was found to be 60,000 (Nakos and Mortenson, 1971).

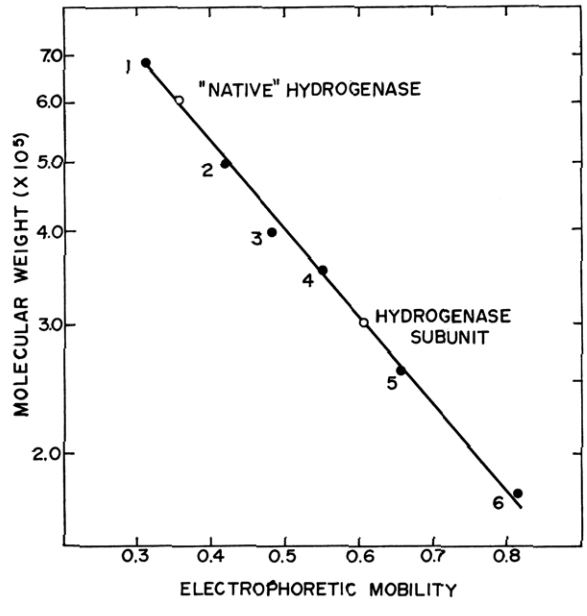

FIGURE 3: Molecular weight estimation of the polypeptide chain of hydrogenase. The marker proteins were (1) bovine serum albumin, (2) fumarase, (3) aldolase, (4) glyceraldehyde phosphate dehydrogenase, (5) chymotrypsinogen, and (6) myoglobin. (0) indicates the position of the native hydrogenase (60,000) and of the hydrogenase subunit (30,000).

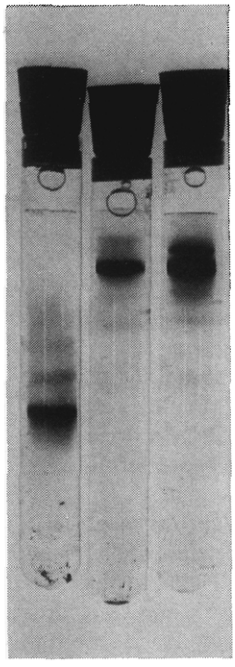

FIGURE 4: Sodium dodecyl sulfate, native and urea-treated hydrogenase samples after electrophoresis. Left: hydrogenase subunits on sodium dodecyl sulfate–polyacrylamide gel prepared as described by Weber and Osborn (1969). Middle: native hydrogenase electrophoresed for 1 hr on a 5% acrylamide gel made 8.0 M in urea. Right: hydrogenase sample dialyzed for 12 hr against 8.0 M urea plus 0.1 M 2-mercaptoethanol before electrophoresis on a 8.0 M urea gel. Urea gels were prepared according to Brewer and Ashworth (1969).

Hydrogenase electrophoresed for one hour on a 5.0% acrylamide gel made in 8.0 M urea (Brewer and Ashworth, 1969) gives only one protein band (Figure 4, middle). This protein band shows some hydrogenase activity when the gels are treated according to Ackrell *et al.* (1969) before staining. This protein appears to correspond with the native enzyme. A partial dissociation of hydrogenase occurs only when the enzyme is dialyzed against 8.0 M urea and 0.1 M 2-mercaptoethanol for 12–24 hr before electrophoresis on gels made 8.0 M in urea (Figure 4, right). The fact that under these conditions only one protein band appears, in addition to the band corresponding to the native enzyme, suggests that the two hydrogenase subunits of equal size have identical isoelectric points.

Amino Acid Composition. Amino acid analysis at two time intervals were performed (Table I). A mean residue weight of 107 and a partial specific volume ($\bar{v}$) of 0.75 (McMeekin *et al.*, 1949) were calculated. The determination of the partial specific

TABLE I: Amino Acid Composition of Hydrogenase from *C. pasteurianum*.

Amino Acid	Time of Hydrolysis: 24 hr (mole/mole)	Time of Hydrolysis: 48 hr (mole/mole)	Mean Value (mole/mole)	Nearest Interger[a]
Aspartic acid	51.8	54.6	53.20	53
Threonine[b]	34.2	30.8	32.50	38
Serine[b]	26.2	23.4	24.80	28
Glutamic acid	22.4	24.2	23.30	23
Proline[c]	15.3	18.0	16.60	18
Glycine	35.2	36.0	35.60	36
Alanine	51.7	49.0	50.30	50
Valine[c]	57.7	58.0	57.80	58
$^1/_2$-Cystine[d]	3.8	3.5	3.70	4[e]
Methionine	14.4	14.1	14.25	14
Isoleucine[c]	54.4	52.0	53.35	54
Leucine[c]	48.7	50.5	49.60	50
Tyrosine	23.0	21.6	22.30	22
Phenylalanine	23.2	24.6	23.90	24
Lysine	51.7	47.8	49.75	50
Histidine	13.0	14.0	13.50	14
Arginine	16.0	15.8	15.90	16
Tryptophan[f]				0

[a] A molecular weight of 60,000 is used for the native enzyme. This value was obtained from gel filtration (Nakos and Mortenson, 1971) and from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2). [b] Values were obtained by extrapolation to a zero hydrolysis time. [c] The highest values are used. [d] Half-cystine was measured as cysteic acid in performic acid (Hirs, 1956; Moore, 1963) and dimethyl sulfoxide (Spencer and Wold, 1969) oxidized samples. [e] This value is corrected for the 90% recovery of the standards in the oxidation procedure. [f] Tryptophan was estimated spectrophotometrically (Bencze and Schmid, 1957), colorimetrically (Spies and Chambers, 1949), and by the acid–ninhydrin reagent (Gaitonde and Dovey, 1970).

volume does not take into account the iron and the acid-labile sulfide present in the native protein. Spectrophotometric determination of tryptophan (Bencze and Schmid, 1957), in the intact protein, yields a value of zero tryptophan residues per molecule of hydrogenase. The method of Spies and Chambers (1949) yields a value of 0.35 residue whereas the method of Gaitonde and Dovey (1970) gives a value of 0.86 residue. However, tryptophan determination by the latter method is, to sume extent, subject to interference by tyrosine present in the protein. From the above results a zero tryptophan value is assigned to hydrogenase.

Elemental Analysis.[2] The elemental composition of a hydrogenase sample from *C. pasteurianum* is (in per cent): C, 48.5; H, 7.4; N, 14.4; S, 1.2.

Carbohydrate Content. The phenol–H_2SO_4 method (Dubois *et al.*, 1956) and the anthrone test (Scott and Melvin, 1953) give zero values for total sugar content. The tests for reducing sugars, pentose sugars, and deoxy sugars are also negative.

[2] The elemental analysis of hydrogenase was carried out by the Microanalytical Laboratory of the Department of Chemistry, Purdue University, Lafayette, Ind.

TABLE II: Dissociation of Iron from Hydrogenase during Dialysis against 0.1% Sodium Dodecyl Sulfate Plus 0.1% 2-Mercaptoethanol.

Dialysis Time (hr)	Direct Dialysis: nmoles/ml	Direct Dialysis: %	Pretreatment[a]: nmoles/ml	Pretreatment[a]: %
0	280	100	112	100
2	125	45	62	55
4	125	45	48	43
6	130	47	26	24

[a] Hydrogenase samples were treated for 4 hr with 2.0% sodium dodecyl sulfate plus 1% 2-mercaptoethanol before dialysis against 0.1% sodium dodecyl sulfate plus 0.1% 2-mercaptoethanol.

Effects of Sodium Dodecyl Sulfate and Urea on Hydrogenase. Hydrogenase is immediately inactivated by 0.1% of sodium dodecyl sulfate in the reaction mixture under anaerobic conditions. In a separate experiment when hydrogenase is dialyzed against 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol, about 50% of the iron dissociates from the protein (Table II). This increases to about 75% when the enzyme is treated with 2.0% sodium dodecyl sulfate and 1.0% 2-mercaptoethanol prior to dialysis (Table II).

Hydrogenase retains its full activity after standing for 6 hr in 4.0 M urea under anaerobic conditions (Figure 5). In 6.0 M urea, under the same conditions, hydrogenase activity declines after 2 hr but it still retains about 25% of its initial activity after 6 hr. The addition of 0.1 M 2-mercaptoethanol to the incubation mixture enhances hydrogenase inactivation (Figure 5).

Dialysis of hydrogenase samples in 4.0 M urea, under anaerobic conditions, results in loss of activity with concomitant losses of iron and acid-labile sulfide (Figure 6). After 4- to 6-hr dialysis the protein still contains the equivalent of 1.0 iron and 1.0 acid-labile sulfide per molecule.

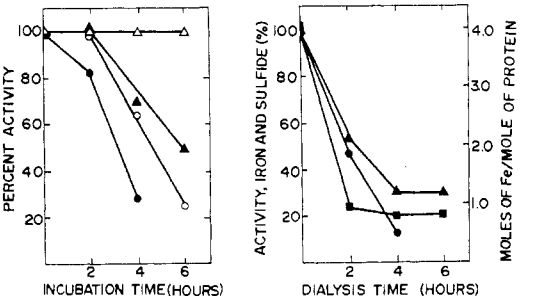

FIGURE 5: Hydrogenase activity in the presence of urea and under anaerobic conditions. Urea (4.0 M) with (▲) and without (△) 0.1 M 2-mercaptoethanol. Urea (6.0 M) with (●) and without (○) 0.1 M 2-mercaptoethanol. The urea solutions were made in air-free 0.05 M Tris-HCl (pH 8).

FIGURE 6: Losses of activity (●), iron (▲), and acid-labile sulfide (■) from hydrogenase upon dialysis. The enzyme (3–5 mg/ml) was dialyzed against 4.0 M urea made in air-free 0.05 M Tris-HCl (pH 8).

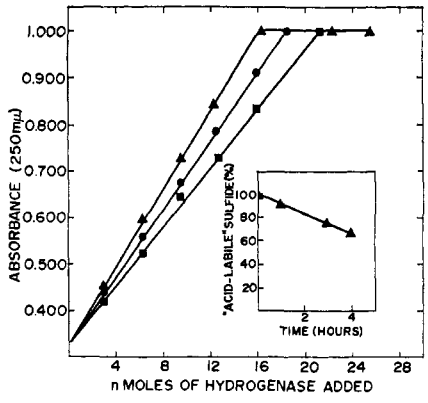

FIGURE 7: Titration of hydrogenase with PMB. PMB (200 nmoles) in 2.0 ml of buffer (0.005 M glycylglycine and 0.045 M Tris-HCl, pH 8) solution was rendered anaerobic (▲) in a cuvet fitted with a rubber serum stopper. Portions (3.2 nmoles) of hydrogenase were added to both the blank and the experimental cuvet and the absorbance at 250 mμ was recorded. The same experiment was repeated, under aerobic conditions, after exposing the enzyme to air for 1 (●) and 3 (■) hr. The amount of acid-labile sulfide in the aerated hydrogenase samples was also measured (insert).

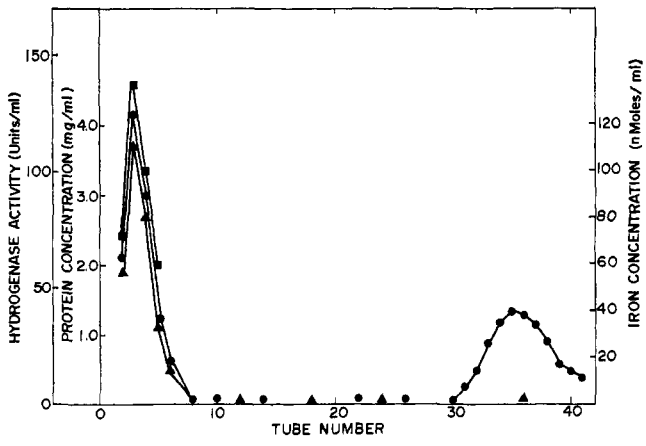

FIGURE 8: Elution of *o*-phenanthroline-treated hydrogenase from a G-25 column. Hydrogenase (0.50 μmole) in 2.5 ml was treated with 6.0 μmoles of *o*-phenanthroline and 10 μmoles of dithionite. The mixture was stirred anaerobically for 0.5 hr before it was added to the column. Fractions of 2.70 ml were collected anaerobically. Under these conditions the millimolar extinction coefficient for the ferrous *o*-phenanthroline complex was 10.5. (▲–▲) Protein concentration, (■–■) activity, and (●–●) iron concentration.

Reaction with Sulfhydryl Reagents. C. pasteurianum hydrogenase was titrated with PMB as described by Boyer (1954) with the results shown in Figure 7. Based on the absorbance change at 250 mμ, the results indicate that approximately 12.5 moles of PMB react with 1.0 mole of the enzyme. Similar results are obtained when the reaction is carried out in 2.0% sodium dodecyl sulfate solution. Since 2 moles of PMB react with 1.0 mole of sodium sulfide (Lovenberg *et al.*, 1963) and since hydrogenase contains 4 moles each of acid-labile sulfide (Nakos and Mortenson, 1971) and half-cystine residues (Table I) per mole of protein, 12.0 moles of PMB should react with 1.0 mole of hydrogenase. These results (Figure 7) would indicate that all the sulfur in hydrogenase (except that in methionine) occurs in a form that can react with PMB without prior reduction and is, therefore, not present as disulfide. When the protein sample is aerated for 1–3 hr and titration is then carried out aerobically, a decrease in the number of PMB-titratable groups is observed. After 1- and 3-hr aeration 10.8 and 9.5 groups, respectively, are detected. Analysis for acid-labile sulfide in hydrogenase samples shows 25% less acid-labile sulfide (about one S^{2-} group per molecule) in samples aerated for 3 hr than in untreated samples (insert in Figure 7). This will decrease the number of PMB-titratable groups from 12 to 10. The value of 9.5 groups found above agrees with the observed decrease in acid-labile sulfide. The titration of hydrogenase with sodium mersalyl requires about 11.7 moles of sodium mersalyl/mole of hydrogenase. This number agrees with the PMB titration results. Similar results are obtained when hydrogenase is titrated with DTNB.

Effects of Iron Chelating Agents and Mercurials on Hydrogenase. Hydrogenase, in the presence of dithionite, reacts with *o*-phenanthroline to give the characteristic color of the ferrous iron *o*-phenanthroline complex (λ_{max} 512 mμ). The amount of color produced is equivalent to 2 moles of iron/mole of protein and yet *o*-phenanthroline added in 8- to 40-fold molar excess has no effect on hydrogenase activity (Table III).

When the *o*-phenanthroline–hydrogenase "complex" is chromatographed on a Sephadex G-25 column two colored bands are resolved (Figure 8). The first band, moving with the void volume of the column, is protein and the second band corresponds to the ferrous *o*-phenanthroline complex. The protein band has hydrogenase activity with a specific activity equal to that of the native enzyme and contains two iron atoms per molecule neither of which reacts with either *o*-phenanthroline or Tiron. Its millimolar extinction coefficient, at λ_{max} 400 mμ, is equal to that of the native enzyme and upon anaerobic titration with PMB it shows the presence of 12 titratable SH equiv. This represents a different case from the one recently reported by Hong and Rabinowitz (1970) concerning the all-or-none reconstitution of clostridial ferredoxin. In their studies partial loss of iron and acid-labile sulfide from ferredoxin was accompanied by a comparable reduction in its specific activity and absorbance at 390 mμ. When hydrogenase reisolated after *o*-phenanthroline treatment (hydrogenase with two iron atoms and four acid-labile sulfide groups) is treated anaerobically with 30- to 50-fold molar excess sodium mersalyl all of its iron reacts with Tiron but not with *o*-phenanthroline.

TABLE III: Effect of *o*-Phenanthroline and Sodium Mersalyl on Hydrogenase Activity.

Compound	Concn (moles/mole of Protein)[a]	Inhibn[b] (%)
o-Phenanthroline[c]	8.15	0
	16.40	0
	40.00	0
Sodium mersalyl	8.15	40
	12.20	70
	24.40	83

[a] Each vessel also contained (in micromoles) per 2.0 ml of total volume: Tris-HCl pH 8 buffer, 75.0; methyl viologen, 1.0; dithionite, 20. Each mole of protein contains 4 moles of iron and 12 SH equiv. [b] Similar results are obtained with the methylene blue assay. [c] α,α′-Dipyridyl and sodium azide also do not inhibit hydrogenase activity at concentrations of 12 moles/mole of protein.

TABLE IV: A Summary of Some Physical and Chemical Properties of Hydrogenase from *C. pasteurianum*.

Molecular weight	60,000
Polypeptide chains	2
Isoelectric point	5
Acid-labile sulfide (moles/mole)	4
Iron (moles/mole)[a]	4 (or 2)[a]
Half-cystine (moles/mole)	4
Methionine (moles/mole)	14
SH equivalents (moles/mole)[b]	12
Total sulfur (%)	1.2 ± 0.01
ϵ_{mM}[c]	8
Turnover number	3,000,[d] 15,000[e]
$\bar{v}$	0.75

[a] Atomic absorption determinations for other metals in hydrogenase show trace amounts of Zn and nondetectable amounts of K, Ca, mg, Cu, and Mo. *o*-Phenanthroline treatment removes two iron atoms without loss of activity. [b] The number of PMB-titratable groups. [c] In $l \times cm^{-1} \times mmole^{-1}$; λ_{max} at 400 mμ. [d] In the methyl viologen assay. [e] In the methylene blue assay.

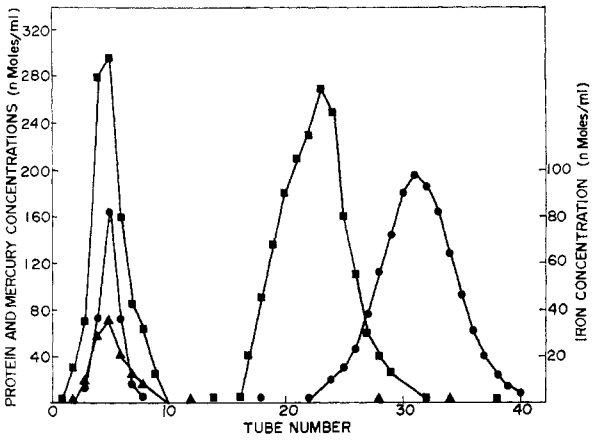

FIGURE 9: Elution of mersalyl-treated hydrogenase from a G-25 column. Hydrogenase (0.61 μmole) in 3.0 ml was treated successively with 14.6 μmoles of sodium mersalyl, 10 μmoles of dithionite, and 7.5 μmoles of *o*-phenanthroline. The mixture was stirred anaerobically for 0.5 hr before it was applied to the column. Fractions of 2.70 ml were collected. (▲–▲) Protein concentration, (■–■) mercury concentration, and (●–●) iron concentration. Under these conditions the millimolar extinction coefficient for the ferrous *o*-phenanthroline complex was 10.5.

Hydrogenase treated anaerobically for 20 min with 24-fold molar excess sodium mersalyl losses about 80% of its activity (Table III). Excess 2-mercaptoethanol added 20 min after the addition of mersalyl does not reverse the inhibition. In a control the mercury of the mersalyl is first complexed with 2-mercaptoethanol and then added to hydrogenase. There is no inhibition which indicates that mersalyl inhibits hydrogenase only through reaction of its mercury with the SH and/or S^{2-} groups of the protein. In a separate experiment hydrogenase was treated for 20 min with 24 molar excess mersalyl followed by treatment with dithionite and *o*-phenanthroline and the mixture was fractionated on a Sephadex G-25 column (Figure 9). Only the equivalent of one iron atom remained with the protein. In all the protein fractions about 4 moles of mercury was found associated with each mole of protein. This suggests that all the acid-labile sulfide is removed from the protein by the mersalyl treatment and mersalyl is bound to the 4 free SH groups.

Recently (Kleiner and Burris, 1970) it has been reported that a significant activation of a partially purified hydrogenase from *C. pasteurianum* occurred when molybdenum (a specially prepared solution) was added to the enzyme. When hydrogenase (this paper) was treated with molybdenum prepared by the procedure of Kleiner and Burris (1970), we were unable to obtain any activation with molar concentrations of molybdenum one to six times the molar concentration of hydrogenase. Hydrogenase preparations used in our studies always have a specific activity of 40–50 μmoles of H_2 evolved/mg per min. This specific activity is significantly higher than that reported for their molybdenum activated enzyme (about 13 μmoles of H_2 evolved per mg per min) and yet our hydrogenase contains no molybdenum.

A summary of some physical and chemical properties of hydrogenase from *C. pasteurianum* is given in Table IV.

Hydrogenase Iso Enzymes. Ackrell *et al.* (1966), Kidman *et al.* (1969), and Kleiner and Burris (1970) have reported that crude extracts from *C. pasteurianum* or partially purified hydrogenase from the same organism shows three to five hydrogenase activity bands after electrophoresis on polyacrylamide gels. These were interpreted to be iso enzymes of hydrogenase (mol wt 55,000).

In the course of the purification of hydrogenase from *C. pasteurianum* (Nakos and Mortenson, 1971) crude extracts also showed three to five activity bands after electrophoresis, depending on the amount of crude protein electrophoresed. The number of active bands decreased to one upon purification of hydrogenase even though little loss in total activity occurred. This suggests that artifacts resulting from protein–protein interactions are occurring on the polyacrylamide gel during electrophoresis and are magnified by the high sensitivity of the hydrogenase assay (Ackrell *et al.*, 1966). To test this hypothesis, hydrogenase was removed from the crude extract (Nakos and Mortenson, 1971) omitting only the heat treatment step. All protein fractions other than hydrogenase were collected and shown to have no hydrogenase activity when tested according to Ackrell *et al.* (1966). When purified hydrogenase (mol wt 60,000) was mixed with the inactive fractions either alone or in combination and the mixture was electrophoresed, the multiplicity of activity bands shown in the crude extract reappears. An explanation for this is that small amounts of hydrogenase associate with proteins of the crude extract and these associations have different electrophoretic mobilities. This phenomenon is seen clearly during the final purification step of hydrogenase where there is only one protein in addition to hydrogenase. The molecular weight of the second protein is 48,000–50,000 and it follows hydrogenase on elution of the mixture from Sephadex G-100 (Nakos and Mortenson, 1971). Figure 10 shows what happens when the protein fractions from this elution profile are electrophoresed, tested for hydrogenase activity, and then stained for protein. Fraction I shows only one protein (1) and one activity band (1A). This protein band corresponds to pure hydrogenase. Fractions II and III show two protein bands and two activity bands but even though the protein in the lower band increases with increasing tube number its corresponding activity band remains almost the same in intensity. Finally fraction IV contains only the second protein and shows no activity

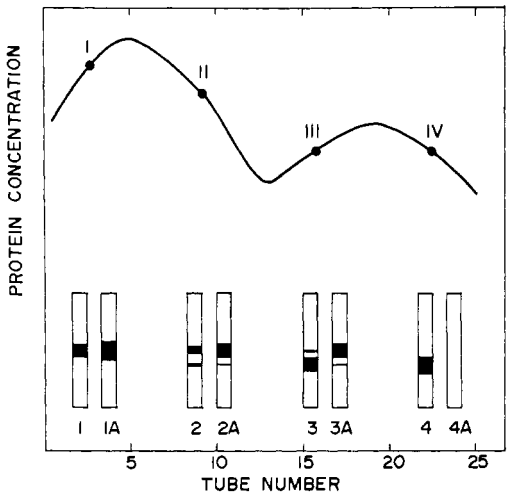

FIGURE 10: Hydrogenase protein (1, 2, 3, and 4) and activity (1A, 2A, 3A, and 4A) band profiles obtained on electrophoresis of protein fractions eluted from a sephadex G-100 column. The gels were tested for hydrogenase activity (Ackrell *et al.*, 1966), stained for protein with Amido Black and destained in 7.0% acetic acid.

band. If the second protein band corresponded to another form of hydrogenase, according to the hydrogenase iso enzymes theory, its activity should have been proportional to its protein concentration. When these protein fractions are tested manometrically for hydrogen evolution, no activity peak corresponding to the second protein peak is observed. In addition, combination of the second protein with hydrogenase does not stimulate hydrogen evolution.

Discussion

Hydrogenase is golden yellow with an extinction coefficient at 400 mμ of about 8000.

The electron spin resonance spectrum obtained suggests that hydrogenase has some properties in common with other iron–sulfur proteins. For example, the signal(s) is in the 1.94 range and is temperature sensitive. It differs from other ferredoxin in that the native form already has the signal which it then loses on oxidation and regains on incubation under H_2 or addition of dithionite.

Hydrogenase activity is very resistant to high concentrations of urea and little dissociation into subunits occurs on urea treatment. The most effective way for dissociating hydrogenase into subunits is a prolonged treatment of the enzyme with 2.0% sodium dodecyl sulfate, in the presence of 2-mercaptoethanol, followed by carboxymethylation. This prevents the reassociation of the subunits and suggests a role for cysteine residues in association.

Dialysis of the enzyme against either 4.0 M urea or 0.1% sodium dodecyl sulfate or treatment with sodium mersalyl results in loss of color, inactivation, and simultaneous loss of iron and acid-labile sulfide. This suggests involvement of both iron and sulfide in the chromophore and at the catalytic site of the enzyme.

Hydrogenase contains a relatively high number of acidic and neutral amino acids as well as lysine residues. This is consistent with its isoelectric point of about 5.0 (Nakos and Mortenson, 1971) and its relatively weak adsorption on DEAE-cellulose during purification. Hydrogenase has 4 half-cystine residues, 4 acid-labile sulfide groups, and 14 methionine residues per molecule. This, based on a molecular weight of 60,000 for hydrogenase, gives a total sulfur content of 1.18%, which is in agreement with the value of 1.2% from the elemental analysis of hydrogenase.

The data obtained from mercurial and DTNB titrations indicates that the cysteine residues of the protein are not involved in disulfide bridges. The decrease in the amount of total acid-labile sulfide of the protein upon oxidation probably reflects its oxidation to a form that does not dissociate from the protein nor react in the sulfide analysis procedure.

Two of the iron atoms of the isolated hydrogenase are easily removed without loss of enzymatic activity. This iron may be contamination. However, the fact that hydrogenase is always isolated with four iron atoms suggests that the "extra" two iron atoms are required for other function(s), perhaps participation in structural features of the enzyme unrelated to its enzymatic activity. The presence of 12 titratable SH equiv after the removal of two of the four iron atoms indicates that none of the acid-labile sulfide groups is removed when the iron is removed.

Since we find no molybdenum associated with hydrogenase of high specific activity (50 μmoles/mg per min) nor any effect of molybdenum salts on this activity, we must conclude that it is not a constituent of hydrogenase. Prior demonstration of molybdenum stimulation by others resulted in a increase in specific activity from 6 to 13 μmoles per mg per min. Possibly the molybdenum preparation used reactivated inactive hydrogenase.

If hydrogenase is an iso enzyme as suggested by studies with the crude enzyme, it should show the same behavior when purified. It does not. Apparently small amounts of hydrogenase bind to proteins in the crude extract and separate from the main unbound hydrogenase when electrophoresed. This results in multiple hydrogenase activity bands. There is the possibility that hydrogenase in the bacterial cell is involved in more than one metabolic function and under these circumstances it may be associated rather strongly with other proteins. During purification of the enzyme these complexes are dissociated.

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Role of Magnesium and Calcium in the First and Second Contraction of Glycerin-Extracted Muscle Fibers*

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ABSTRACT: The role of Mg, Ca, and ATP in isometric contraction of well-washed, glycerin-extracted skeletal muscle was studied. Two contractions were obtained: (1) a rapid "first" contraction dependent only on Mg and ATP at [Ca^{2+}] below threshold for ATPase activation (less than 10^{-8} M Ca^{2+}); (2) a much slower "second" contraction requiring [Ca^{2+}] above 3×10^{-7} M in addition to Mg and ATP. The initial speed of the first contraction is related to [$MgATP^{2-}$] and follows a Langmuir adsorption isotherm. The speed is maximal at zero time. These results suggest that the initial event is the binding of [$MgATP^{2-}$] to the fiber. The fibers relax spontaneously when [Ca^{2+}] is below 10^{-7} M and after this relaxation a slower "second" contraction occurred upon raising the [Ca^{2+}] to above 3×10^{-7} M. The rate of the second contraction is related to the [Ca^{2+}] in the range of 10^{-7}–10^{-5} M, a range required for activation of fiber ATPase suggesting that Ca^{2+} activates ATPase and the second contraction is controlled by the rate of ATP hydrolysis and the subsequent binding of new MgATP. When Ca is present in the initial contraction mixture, the fibers remain contracted because MgATP is turned over continuously. The fact that the two contractions occur under different conditions may explain the controversies concerning the requirements for Ca^{2+} and for ATP hydrolysis in muscular contraction.

It is generally believed that the release of calcium by the sarcoplasmic reticulum is an intermediate step in the excitation-coupling mechanism of muscular contraction (Sandow, 1965; Nayler, 1967) and Ca^{2+} has been reported to be a requirement for the contraction of glycerin-extracted skeletal muscle (Seidel and Gergely, 1963; Filo *et al.*, 1965; Portzehl *et al.*, 1965; Schädler, 1967). Magnesium (Mg) and ATP were demonstrated early (Szent-Györgyi, 1946, 1947; Bowen, 1951) to be also a requirement for the contraction of glycerin-extracted muscle and this requirement is now generally accepted. The requirement for Ca is still controversial because in fibers which had been extracted for more than 12 weeks, Mg (and ATP) will cause a maximal contraction which is not enhanced by Ca (Embry and Briggs, 1966). Briggs and King (1962) who studied fibers which were extracted for more than 2 weeks in glycerine plus deoxycholate also concluded that there is no relationship between the Ca^{2+} in their contraction mixtures and the contractions. These authors even treated their chemicals with Chelex cationic exchange resin to remove traces of Ca and this treatment did not affect their contractions. They reported that they were able to reduce the Ca contamination in their contraction solutions to less than 7×10^{-9} M, and calculated that the free Ca concentration was probably less than 7×10^{-10} M. Watanabe *et al.* (1964) have also reported that glycerinated extracted fibers can be contracted completely in the absence of Ca.

Takahashi *et al.* (1965) studied the contraction of glycerin-extracted isolated sarcomeres which were resuspended several times and concluded that the MgATP complex is essential for these contractions and that Ca is nonessential. Kuribayashi (1969) found that Ca is not essential for ATP (Mg) contractions of glycerinated taenia coli muscle. This conclusion agrees

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