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PURIFICATION AND PROPERTIES OF THE MEMBRANE-BOUND HYDROGENASE FROM *PROTEUS MIRABILIS*

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Summary

The cytoplasmic membrane-bound hydrogenase of the facultative anaerobe, *Proteus mirabilis*, has been solubilized and purified to homogeneity. The purified enzyme exhibited a maximal specific activity of about 780 μmol H_2 oxidized/min per mg protein (benzyl viologen reduction). The hydrogenase has a molecular weight of 205 000 and is composed of two subunits with a molecular weight of 63 000 and two of 33 000. The absorption spectrum of the enzyme was characteristic of non-heme iron proteins. The millimolar extinction coefficients at 400 and 280 nm are 106 and 390, respectively. The hydrogenase has about 24 iron atoms and 24 acid-labile sulfide atoms/molecule. Amino acid analyses revealed the presence of 39 half-cystine residues/molecule and a preponderance of acidic amino acids. The hydrogenase in its oxidized form exhibits an EPR signal of the HiPIP-type with g values at 2.025 and 2.018. Upon reduction with either dithionite or H_2 the signal disappears; no other signals were detectable.

Introduction

The enzyme hydrogenase which catalyzes the oxidation of molecular hydrogen as well as the reverse reaction, has been detected in a large number of bacteria and algae [1]. Most of the reports about the purification and characterization of hydrogenase deal with preparations from strictly anaerobic bacteria [2—9]. Less is known about hydrogenase of aerobic and facultative anaerobic bacteria. A soluble, as well as a membrane-bound, hydrogenase were purified from the aerobic hydrogen bacterium, *Alcaligenes eutrophus*, [10—12]. No reports on purified hydrogenase from facultative anaerobic bacteria

have yet been presented. A few data on a partially purified hydrogenase from *Proteus vulgaris* are available [13].

In *Proteus mirabilis* as in other gas-producing species of the *Enterobacteriaceae*, hydrogenase, formate dehydrogenase and one or more electron carriers are part of a cytoplasmic, membrane-bound multienzyme complex, the so-called 'formate hydrogenlyase system', which is concerned with the production of CO_2 and H_2 during anaerobic growth [14—18]. Also the reverse action of hydrogenase, coupling oxidation of hydrogen to anaerobic respiration, has been demonstrated in *P. mirabilis* by De Groot and Stouthamer [19]. The latter authors also described some regulation aspects of the formation of hydrogenase and formate hydrogenlyase in this bacterium [18,20].

In this paper, a reliable purification procedure and data on molecular, structural and spectral properties of hydrogenase from *P. mirabilis* are described.

Materials and Methods

Chemicals. The chemicals used were: benzyl viologen (British Drug Houses, Poole, U.K.); deoxyribonuclease, ribonuclease and the molecular weight markers RNA polymerase, bovine serum albumin, hen egg albumin and chymotrypsinogen A (Boehringer Mannheim, F.R.G.); acrylamide, *N,N'*-methylenebisacrylamide (Serva, Heidelberg, F.R.G.); *Clostridium pasteurianum* ferredoxin type IV (Sigma, Saint Louis, U.S.A.); phenyl-Sepharose CL-4B, DEAE-Sephadex A50 and Sepharose 6B (Pharmacia, Uppsala, Sweden). All other chemicals were from Merck (Darmstadt, F.R.G.).

Organism and cultivation. P. mirabilis strain S503 was grown anaerobically in complex medium in a pH-auxostat as described previously [21]. After harvesting, the cells were washed with 25 mM Tris-HCl buffer (pH 7.2), divided into portions of about 100 g (wet weight) and stored at −80° C.

Hydrogenase assay. Hydrogenase activity was measured manometrically, following the uptake of H_2 at 37° C, with benzyl viologen as electron acceptor. The unit of activity was defined as μmol H_2 oxidized/min. The reaction mixture in the Warburg vessels contained in 3.0 ml: 50 mM Tris-HCl buffer (pH 7.2), 5.3 mM benzyl viologen, 1 mg bovine serum albumin and 0.10—0.15 unit enzyme sample. The shaking vessels were flushed for 15 min with H_2 and the reaction was started, after 10 min temperature equilibration, by tipping the benzyl viologen solution from the side arm into the main compartment.

Hydrogenase purification. Unless indicated otherwise all purification steps were performed at 0—4° C. No special anaerobic precautions were taken. The buffer used throughout the purification procedure was 25 mM Tris-HCl (pH 7.2).

Preparation of the crude membrane fraction. A 100 g portion of cells was thawed and suspended in a final volume of 280 ml Tris buffer, containing 10 μg/ml RNAase and 10 μg/ml DNAase. The cells were then broken by two passes at $1.4 \cdot 10^8$ Pa through a Ribi cell-fractionator (Sorvall) and centrifuged for 10 min at 10 000 × *g* to remove whole cells. Next the remaining supernatant was centrifuged for 2 h at 250 000 × *g* and the pellet was resuspended in Tris buffer and diluted to a protein concentration of 8—12 mg/ml with the same buffer.

Solubilization of hydrogenase. Hydrogenase and other cytoplasmic mem-

brane proteins were released into an aqueous solution by a 2-fold extraction of the membranes with *tert*-amyl alcohol at −4° C [22]. The aqueous solution was dialyzed for 40 h against three succesive volumes of 12 l Tris buffer. A precipitate formed during dialysis was removed by centrifugation at 40 000 × *g* for 30 min and discarded. The enzyme was then precipitated with $(NH_4)_2SO_4$ in two steps: first at 60% and secondly at 100% saturation. The pellets were collected by centrifugation at 23 000 × *g* for 30 min, resuspended in 50 ml Tris buffer and dialyzed for 16 h versus 12 l of the same buffer. After dialysis a slight precipitate was removed by centrifugation and discarded. The remaining supernatant contained the solubilized membrane proteins, including hydrogenase.

First phenyl-Sepharose CL-4B chromatography. The solubilized membrane proteins were applied to a phenyl-Sepharose CL-4B column (1.6 × 20 cm), pre-equilibrated with Tris buffer. After unbound protein had been washed out with Tris buffer, elution of hydrogenase activity in one single protein peak was achieved with 10 mM imidazole buffer (pH 7.2; flow rate, 25 ml/h).

DEAE-Sephadex chromatography. The protein peak was adsorbed on a DEAE-Sephadex A50 column (2.6 × 15 cm), pre-swollen in Tris buffer. Before elution was started with a linear KCl gradient (0—0.4 M in Tris buffer; total volume 240 ml; flow rate, 15 ml/h; 2 ml/fraction), 2 column volumes of Tris buffer were passed through the column to remove the imidazol buffer. Hydrogenase was eluted at a Cl^- concentration of approx. 0.2 M.

Sepharose 6B chromatography. The most active fractions were combined, concentrated by ultrafiltration through an Amicon Diaflo Ultrafilter UM20 and then loaded on a Sepharose 6B column (2.6 × 100 cm). The column was pre-equilibrated and eluted with Tris buffer (flow rate, 18 ml/h; 2.4 ml/fraction).

Second phenyl-Sepharose CL-4B chromatography. The most active hydrogenase fractions from the previous step were combined and adsorbed at the top of a second phenyl-Sepharose CL-4B column (0.9 × 15 cm), which was pre-equilibrated with Tris buffer. After the column had been washed with 1 column volume of Tris buffer, elution was performed with a linear gradient of 0—60% (v/v) ethylene glycol in Tris buffer (total volume 192 ml; flow rate, 12 ml/h; 2 ml/fraction). Hydrogenase was eluted at approx. 45% ethylene glycol. The hydrogenase-containing fractions were combined, dialyzed extensively against Tris buffer, concentrated by ultrafiltration to a protein concentration of 1—2 mg/ml and stored at −80°C or in liquid N_2.

Polyacrylamide gel elelctrophoresis. Analytical disc gel electrophoresis was performed according to Maurer [23]. Protein was stained with a 1% (w/v) amido black solution in 7% (v/v) acetic acid. To test the gels for hydrogenase activity, they were immersed in an H_2-saturated solution of 40 mM benzyl viologen in 50 mM Tris-HCl buffer (pH 7.2). After about 15 min, a diffuse violet zone of reduced benzyl viologen arose at the site(s) of hydrogenase activity.

The subunit structure of hydrogenase was estimated on a sodium dodecyl sulfate (SDS)-containing gel system [24]. Before electrophoresis, hydrogenase samples were incubated in 1% (w/v) SDS at 100° C for 5 min in the presence or absence of 1% (v/v) 2-mercaptoethanol. For molecular weight determinations of subunits, the following calibration proteins were used: chymotrypsinogen A,

RNA polymerase, hen egg albumin and bovine serum albumin.

Determination of molecular weight and sedimentation coefficient. The molecular weight and the $s_{20,w}$ value of purified hydrogenase were determined at 20°C in a Beckman model E analytical ultracentrifuge by conventional sedimentation equilibrium and sedimentation velocity, respectively [25]. Protein concentrations of 0.1—0.4 mg/ml were used in these determinations.

Spectral studies. The absorption spectrum of hydrogenase was measured with a Varian SuperScan 3 recording spectrophotometer. Electron spin resonance measurements were performed on a Varian E-9 spectrometer as described by Albracht et al. [26].

Amino acid analysis. Protein samples were dialyzed against 0.1 M NH_4HCO_3 (pH 8.0), lyophilized, hydrolyzed at 110°C in 6 M HCl under vacuum for 24 h and analyzed with a Biotronik LC 6000 automatic amino acid analyzer. The methionine and half-cystine content were determined on a performic acid-oxidized sample [27]. Tryptophan was estimated spectroscopically by the method of Edelhoch [28].

Iron and acid-labile sulfide. Total iron was determined by atomic absorption with an Unicam SP1900 spectrophotometer. Acid-labile sulfide was determined by the method of Suhara et al. [29].

Protein determination. Protein was measured by the method of Lowry et al. [30] or Hartree [31], using dried bovine serum albumin as standard.

Results

Purification of hydrogenase

The results of the procedure developed for the solubilization and purification of hydrogenase from *P. mirabilis*, are summarized in Table I. The first hydrophobic chromatography step on phenyl-Sepharose CL-4B with the concentrated solubilized membrane proteins gave a high degree of purification. Most of the protein applied to the column did not adsorb and could be washed out with Tris buffer. After conventional ion-exchange chromatography and gel filtration, the final purification step, which gave pure hydrogenase, was a second hydrophobic interaction chromatography step. The elution pattern of

TABLE I

PURIFICATION OF HYDROGENASE FROM *P. mirabilis*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Spec. act. (units/mg protein)	Recovery of activity (%)
Crude membrane fraction	480	5760.0	1516	0.26	100.0
Solubilized membrane proteins	73	375.8	1210	3.22	79.8
First phenyl-Sepharose CL-4B column	15	21.9	910	41.60	60.0
DEAE-Sephadex A50 column	36	6.6	728	110.30	48.0
Sepharose 6B column	40	2.6	619	238.10	40.8
Second phenyl-Sepharose CL-4B column	36	1.6	477	298.10 *	31.4

* The activity was measured in the H_2 uptake assay at 37°C with 5.3 mM benzyl viologen as the electron acceptor. For the final preparation a maximal activity of 775.1 units/mg was estimated from measurements of the apparent K_m for benzyl viologen.

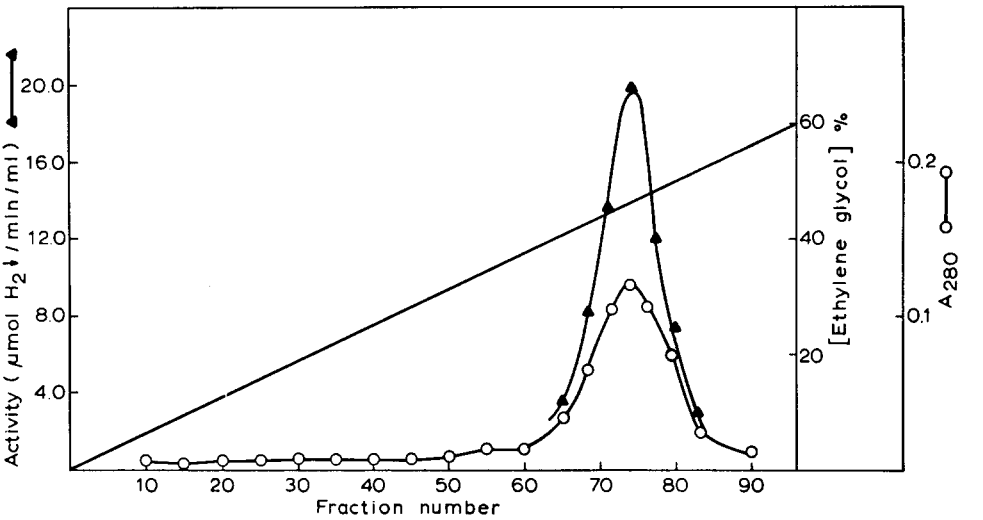

Fig. 1. The elution profile of hydrogenase from the second phenyl-Sepharose CL-4B column (0.9 × 15 cm). About 2.6 mg protein was applied to the column and eluted with a linear gradient of ethylene glycol (0—60%, v/v, in 25 mM Tris-HCl buffer, pH 7.2; 2 ml/fraction; flow rate, 0.2 ml/min).

the column is given in Fig. 1. The specific hydrogenase activity in the eluate now reached 300 units/mg. The conditions employed in the hydrogenase activity measurements do not permit maximum velocity, because of the low benzyl viologen concentration. The apparent K_m for benzyl viologen with solubilized hydrogenase was found to be 9 mM. Taking into account the benzyl viologen concentration of 5.3 mM during hydrogenase measurements, a maximum enzyme activity of 2.6 times the actually measured value can be deduced from Lineweaver-Burk plots, implying a maximum specific hydrogenase activity of about 780 units/mg for the final preparation.

During polyacrylamide gel electrophoresis and analytical ultracentrifugation, the final preparation was revealed to be homogeneous. Membrane-bound, as well as purified, hydrogenase preparations were stable in air and could be stored frozen at −80° C for several weeks with only small losses in activity.

Molecular weight and sedimentation coefficient

The sedimentation coefficient and the molecular weight of the purified hydrogenase were determined in the analytical ultracentrifuge. The $s_{20,w}$ value was found to be 8.68 S. The molecular weight was calculated to be 205 000, assuming a partial specific volume of the hydrogenase molecule of 0.733, which was estimated from the amino acid composition according to Zamyatnin [32]. In order to exclude errors caused by polimerization or aggregation of the enzyme, sedimentation velocity runs were executed at very low protein concentrations (up to 0.1 mg/ml) in the presence and absence of 0.2% deoxycholate. In both cases, the sedimentation patterns pointed to a strictly homogeneous protein sedimentation. The sedimentation coefficient in the presence of deoxycholate was calculated to be 8.3 S. This value does not differ significantly from the $s_{20,w}$ value found in the absence of detergent.

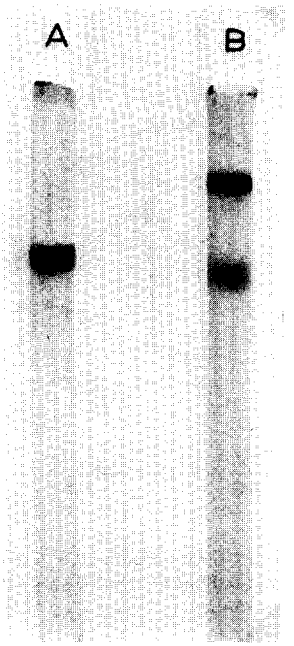

Fig. 2. Polyacrylamide gel electrophoresis with the hydrogenase-containing eluate from the second phenyl-Sepharose CL-4B column. Protein was stained with 1% aminodo black in 7% acetic acid. (A) Analytical electrophoresis according to Maurer [23]. Approx. 30 μg of protein was loaded onto the gel. The separating gel contained 7% acrylamide and was run at pH 8.0. (B) Electrophoresis on a SDS-containing gel [24]. Protein (20 μg) was pretreated with 1% SDS and 1% 2-mercaptoethanol for 5 min at 100°C.

Polyacrylamide gel electrophoresis

Upon polyacrylamide gel electrophoresis under activity-preserving conditions the purified hydrogenase preparation (approx. 30 μg protein/gel) gave a single protein band (Fig. 2A), which exhibited hydrogenase activity as demonstrated by the reduction of benzyl viologen in the presence of H_2. Upon SDS-polyacrylamide gel electrophoresis, the hydrogenase was resolved into two distinct protein bands (Fig. 2B). The molecular weights of the two subunits were approx. 63 000 and 33 000. Densitometric tracings of the SDS gels (not shown) indicate that the molecular ratio of the subunits in the native enzyme is 1 : 1, assuming that the dye-binding capacity is the same in both types of subunits. Therefore, in view of the molecular weight of the native enzyme, it can be concluded that hydrogenase in *P. mirabilis* consists of four subunits, two of 63 000 daltons and two of 33 000 daltons. Omission of 2-mercaptoethanol during SDS treatment and following electrophoresis did not change the protein pattern, which suggests that the subunits are not covalently bound.

Iron and acid-labile sulfide content

Analysis of the purified enzyme for total iron and acid-labile sulfide revealed the presence of 23.2 atoms of iron and 24.0 atoms of acid-labile sulfide/molecule of 205 000 molecular weight (each value is the average of three determinations on different preparations). No interference of protein in the determina-

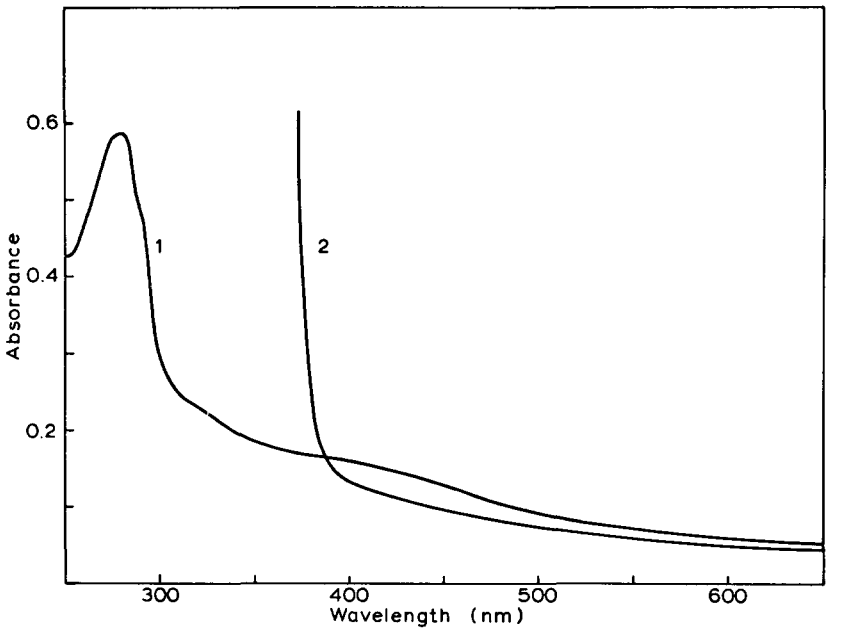

Fig. 3. Absorption spectra of hydrogenase. The protein concentration was 0.31 mg/ml in 25 mM Tris-HCl buffer (pH 7.2). The spectra were taken in a Varian SuperScan 3 recording spectrophotometer. Curve 1, the enzyme as isolated; curve 2, after reduction with a slight excess of dithionite.

tion of iron by atomic absorption was found when standard iron solutions were run in the presence or absence of hydrogenase samples. The reliability of the iron determination was proven by finding 7.4 atoms of iron in a commercial available preparation of ferredoxin type IV from *Cl. pasteurianum* known to contain 8.0 atoms of iron/molecule. The standard Na_2S solutions used in the determination of acid-labile sulfide were calibrated by iodometric titrations.

The absorption spectrum

The absorption spectrum of the purified hydrogenase in the 250—650 nm region (Fig. 3), is in agreement with the iron-sulfur character of this protein, and similar to those reported for other purified hydrogenases [2,4,6]. A broad absorption band around 400 nm was found with a slight shoulder at 325 nm. Addition of a slight excess of dithionite to the isolated enzyme reduced its absorption in the 400—500 nm range. Assuming a molecular weight of 205 000 the millimolar extinction coefficients at 400 and 280 nm were found to be 106 and 390, respectively.

Amino acid composition

The amino acid composition of the purified hydrogenase is shown in Table II. An average number of 39 cysteine residues was found/mol hydrogenase. The enzyme has a preponderance of acidic amino acids with an average ratio of (Asx + Glx)/(Lys + Arg) of about 2.2. This is in agreement with its acidic isoelectric point which has been reported to be 4.75 [22]. Furthermore the

TABLE II

AMINO ACID COMPOSITION OF HYDROGENASE FROM *P. mirabilis*

Two hydrogenase samples were hydrolyzed separately at 110°C in 6 M HCl under vacuum for 24 h. Duplicate analyses were performed on each hydrolysate. The residues/205 000 mol. weight are calculated to the nearest integer.

Amino acid	Residues/205 000 mol. weight
Acidic residues	
Asx	180 ± 15
Glx	212 ± 8
Basic residues	
Lys	115 ± 2
His	65 ± 2
Arg	69 ± 1
Hydroxyl residues	
Thr	91 ± 4
Ser	108 ± 2
Alipathic residues	
Gly	212 ± 3
Ala	118 ± 5
Val	154 ± 9
Ile	107 ± 4
Leu	144 ± 2
Pro	53 ± 5
Met *	13 ± 2
Cys *	39 ± 3
Aromatic residues	
Tyr	68 ± 1
Phe	62 ± 1
Trp **	45 ± 1
Total residues	1855 ± 70

* Values obtained from performic acid-oxidized samples [27].

** Determined spectrophotometrically according to Edelhoch [28].

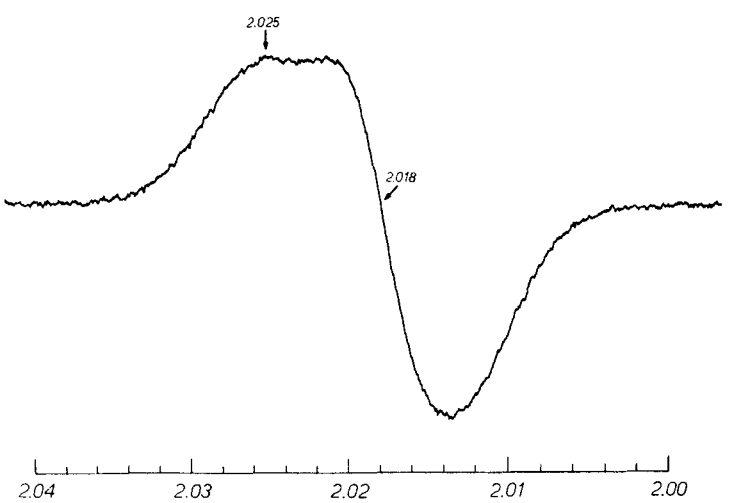

Fig. 4. EPR spectrum of hydrogenase as isolated. The enzyme concentration was about 4 μM. EPR conditions: frequency, 9311 MHz; temperature, 7.5 K; microwave power, 0.2 μW; modulation amplitude 0.63 mT; scanning rate, 1.25 mT/min. The field modulation frequency was 100 kHz. The *g* values of the signal are indicated by arrows. The *g* value scale is at the bottom of the figure.

enzyme has a relative high content of hydrophobic residues, which probably explains its behaviour on the hydrophobic phenyl-Sepharose CL-4B columns.

EPR spectrum

The enzyme in the isolated form exhibited a signal, probably axial, with $g_{//}$ = 2.025 and $g_{\perp}$ = 2.018 (Fig. 4). The signal became visible below a temperature of 30 K. At temperatures lower than 12 K, the signal was no longer temperature sensitive. Addition of ferricyanide did not change the spectrum. On reduction with either dithionite or H_2, the signal disappeared and no other signals were detectable. It can be concluded that the signal is of the HiPIP-type, characteristic of [4Fe-4S] centers in the mono anionic state [33,34]. In view of the 24 atoms of iron and acid-labile sulfide/molecule hydrogenase the EPR spectrum revealed surprisingly slight complexity. There was no loss of enzymic activity of the EPR sample after EPR spectroscopy. Consequently, the lack of complexity cannot be due to damage of the enzyme during EPR measurements. Quantitation of the observed signal showed an intensity equivalent to about 1.3 unpaired electron spins/molecule. Obviously, under the conditions employed so far, not all the ironsulfur centers are in a paramagnetic state.

Discussion

The procedure described in this report affords solubilization and purification of hydrogenase from the facultative anaerobe *P. mirabilis*. Determination of physico-chemical properties of the hydrogenase preparation gave coherent results and indicated that the enzyme was pure. During the purification, no special anaerobic precautions were needed, since the enzyme was found to be stable in the presence of oxygen. This property is in common with hydrogenases purified from *Alcaligenes eutrophus* [10—12], *Chromatium* [7], *Desulfovibrio vulgaris* [5] and *Rhodospirillum rubrum* [8]. The molecular weight of the native enzyme (205 000), determined by sedimentation equilibrium, is in godd agreement with the molecular weight deduced from the subunit structure (192 000). A comparable molecular weight (205 000) was reported by Schneider and Schlegel [10] for the soluble hydrogenase from *A. eutrophus*. A number of hydrogenases in other bacteria have been reported to have about half this molecular weight, 115 000 in *P. vulgaris* [13], 89 000 in *D. vulgaris* [9], 89 500 daltons in *D. gigas* [6] and 100 000 for the membrane-bound hydrogenase in *A. eutrophus* [11]. The hydrogenases described in the latter three reports are composed of two different subunits of which the size is comparable with the size of the subunits in the hydrogenase of *P. mirabilis*. These facts suggest that the enzyme in our preparations may be dimerized. However, neither during the purification, nor during sedimentation velocity studies at low protein concentrations, even in the presence of detergent, was any indication for the presence of a half-size hydrogenase found.

The almost equal amounts of iron and acid-labile sulfide (respectively, 23.2 and 24.0 atoms/molecule of 205 000 daltons), are compatible with the estimated half-cystine content (average, 39 mol/mol enzyme). The millimolar extinction coefficient at 400 nm was found to be 106, which is about twice those reported for the hydrogenases of *D. vulgaris* [5] and *D. gigas* [6], both

containing 12 atoms of iron and acid-labile sulfide. The nature of the iron-sulfur centers in the enzyme of *P. mirabilis* is not yet known, however its iron-sulfur content and the fact that the 12 iron-sulfur atoms in the hydrogenases of *Cl. pasteurianum* and *D. gigas* have been reported to be arranged in three tetrameric iron-sulfur centers [6,35], suggest that there are six tetrameric FeS centers in the hydrogenase of *P. mirabilis*. The low spin intensity and the slight complexity of the EPR signal indicate that hydrogenase in its isolated form, has most probably several EPR-silent iron-sulfur centers. It has been reported by Chen et al. [36] that the hydrogenase of *Cl. pasteurianum* in its reduced form gave 1.66—1.80 spins/mol enzyme and they proposed that one of the three iron-sulfur clusters was EPR silent under this condition. Low spin intensities have been observed also in the EPR signal of the MoFe protein of nitrogenase [37]. Mössbauer experiments have suggested that the EPR signals of this metalloprotein only originate from about 8 of the 24 iron atoms present [38]. An EPR signal very similar to that of hydrogenase from *P. mirabilis* has been observed in the oxidized form of the following iron-sulfur proteins, all of which contain at least four iron and four inorganic sulfur atoms: nitrate reductase A from *Micrococcus denitrificans* [39], super-oxidized Clostridial-type ferredoxins [40], ferredoxin I from *Azotobacter vinelandii* [41] and the hydrogenases from *Chromatium* [7] and *R. rubrum* [8].

Throughout the purification procedure, we used the Peck and Gest assay for hydrogenase activity [16], in which the oxidation of H_2 is coupled to the reduction of benzyl viologen. In a brief survey of other electron acceptors, it was found that, instead of benzyl viologen, methylene blue and methyl viologen could be used too, but NAD^+ failed in the acceptance of electrons from the hydrogenase of *P. mirabilis*. It is a matter of course that, besides the oxidation of H_2, the enzyme must also catalyze the reverse reaction, in which protons are reduced to H_2 on account of the oxidation of a suitable electron donor. In a common assay for this H_2-production activity, the electrons are donated to the enzyme by dithionite with mediation of methyl viologen at pH 8.0. However, this assay appeared to be unsuitable for our enzyme preparations. The measured H_2 production was low and variable, for reasons which are not yet understood. Further investigations on electron donating systems for the H_2 production by the hydrogenase of *P. mirabilis* are now in progress.

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