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CHARACTERIZATION OF THE PERIPLASMIC HYDROGENASE FROM DESULFOVIBRIO GIGAS

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SUMMARY

The hydrogenase of the sulfate-reducer Desulfovibrio gigas has been purified to homogeneity. The pure enzyme shows a specific activity of 90 µmoles H_2 evolved/min./mg protein. Its molecular weight is 89,500 and its is composed of two different subunits (mol. wt. : 62,000 and 26,000) which are not covalently bound. The absorption spectrum of the enzyme is characteristic of an iron-sulfur protein. The millimolar extinction coefficients of the hydrogenase are 46.5 and 170 respectively at 400 and 280 nm. It contains about 12 iron atoms and 12 acid-labile sulfur groups per molecule and the quantitative extrusion of the Fe-S centers of the hydrogenase indicates the presence of 3 Fe_4S_4 clusters. This hydrogenase has 21 half-cystine residues per molecule and a preponderance of aromatic amino-acids.

The enzyme hydrogenase is involved in the reversible activation of molecular H_2. It is unique in that its substrate, gaseous H_2, is the simplest molecule known.

Extensive purification of hydrogenase from different types of microorganisms has been reported in the last few years (1-7). The data reported have established that hydrogenase is an iron-sulfur protein. Two kinds of hydrogenases have been described among the strict anaerobes with different specificities for the electron carriers, cytochrome c_3 (8,9) and ferredoxin (1) though still another hydrogenase of unknown specificity has been reported (3).

The most complete data on physico-chemical properties of hydrogenase have been obtained from the study of highly purified preparations of the enzyme from Clostridium pastorianum (1, 10). This protein is constituted by a single chain and contains 12 half-cystine residues, 12 iron atoms and 12 acid-labile sulfide per molecule of molecular weight 60,500.

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In sulfate-reducing bacteria, a soluble hydrogenase has been isolated from Desulfovibrio vulgaris, Hildenborough (2) and D. gigas (9, 11) whereas a membrane associated enzyme has been purified from D. vulgaris, Miyazaki (4). The former enzyme has been found to exist in a dimer form of molecular weight 60,000 containing 3-4 iron and labile sulfide atoms per molecule and the latter has been reported to be composed of two different subunits (mol. wt. 28,000 and 59,000) and to contain 8 iron and labile-sulfide atoms per molecule (mol. wt. 89,000). When compared to the three clusters (4 Fe- 4 S) containing hydrogenase from C. pastorianum (12) these preparations contain only 1 or 2 Fe_4S_4 centers per molecule and exhibit lower specific activities. It is important to check whether the differences observed are due to a heterogeneous state of these preparations as it was previously the case with C. pastorianum enzyme (1, 13) or not. We present here an improved purification procedure of D. gigas hydrogenase and we report some of its physico-chemical properties.

MATERIALS AND METHODS

Analytical methods

Desulfovibrio gigas was cultivated and harvested as previously described (14).

Enzyme assay. Hydrogenase activity was routinely measured by the H_2 evolution essay (15) with 1 mM methyl viologen as the electron carrier and 15 mM $Na_2S_2O_4$ as the electron donor. The reaction was carried out in Warburg apparatus at 32° C under Ar. Tris-HCl buffer at pH 7.6 (0.05 M) was used in the assay and the preparation of all the reagents which are then degazed and flushed with Ar. All the ingredients except the enzyme were introduced in the main compartment and the reaction was started by tipping in the enzyme from the sidearm. The total volume of the reaction mixture was 3.0 ml.

Protein determination. Protein was determined by the Lowry method (16) using dried bovine serum albumin as standard.

Acrylamide gel electrophoresis. Analytical gel electrophoresis was performed in 7 per cent polyacrylamide gel with Tris-HCl glycine buffer at pH 8.9. Each gel was loaded with 30-50 µg protein and protein bands were revealed with Coomassie blue.

Determination of molecular weight. The molecular weight of purified hydrogenase was determined by analytical ultracentrifugation with a Beckman model E analytical ultracentrifuge. It was estimated at equilibrium sedimentation by meniscus depletion method as developed by Yphantis (17). The partial specific volume was measured using the Paar densitometer. Subunits mo-

lecular weight was estimated by the method of Weber and Osborn (18). The samples were incubated at 37° C for 2 hours in 10^{-2} M phosphate buffer, pH 7.0, 1 % in sodium dodecyl sulfate (SDS) in the presence and absence of 1 % β-mercaptoethanol. The following protein standards were used : bovine serum albumin, ovalbumin, chymotrypsinogen A, soybean trypsin inhibitor and cytochrome c.

Spectrophotometric studies. The UV-visible absorption spectra were measured with a Cary 14 recording spectrophotometer.

Iron and acid-labile sulfide determination. Total iron was determined by the o-phenanthroline method and by atomic absorption spectrometry using an Unicam model SP 1900 spectrometer. Labile sulfide was estimated by the method of Fogo and Popowsky (19) as modified by Suhara et al. (20).

Active site core extrusion. Quantitative extrusion of the F_4S_4 cores of the active site of the hydrogenase of D. gigas was carried out anaerobically in 4 : 1 v/v hexamethylphosphoramide (HMPA)/H_2O medium (50 mM Tris-HCl pH 8.5) using benzenethiol according to Gillum et al. (12).

Amino acid analysis. Amino-acid analysis were performed on hydrogenase samples hydrolyzed for 20 h, 48 h and 70 h at 110° C in 6 M HCl in evacuated, sealed tubes according to the method of Moore and Stein (21). The average was calculated from several analysis. The amino-acid composition was determined with a LKB 3201 amino-acid analyzer. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs (22). Tryptophan content was estimated from the ultraviolet absorption spectrum according to Edelhoch (23).

Purification of hydrogenase. The purification of the hydrogenase from D. gigas was performed under anaerobic conditions at 4° C. Tris-HCl and phosphate buffers pH 7.6 used in this procedure contained 1 mM $Na_2S_2O_4$ as described by Chen and Mortenson (1). The periplasmic location of D. gigas hydrogenase (24) has been utilized to extract the enzyme. Freshly thawed cells of D. gigas (350 g wet weight) were carefully suspended in 80 ml 50 mM Tris-HCl buffer and the bacterial suspension was homogenized with a stirring rod as already reported for the extraction of cytochrome c_3 (14). The suspension was centrifuged at 35,000 x g for 30 min. and the reddish brown supernatant was collected. The extraction was repeated once and the cells were frozen. The wash fraction obtained by combination of the two supernatants was subsequently centrifuged at 120,000 x g for 2 hours and passed through a DEAE-cellulose (DE-52) column (3.5 x 23 cm) equilibrated with 50 mM Tris-HCl buffer. The enzyme which was not retained on DE-52 at this stage was collected by washing the column with 300 ml of the same buffer. The active fraction was passed through a silica gel column (3 x 15 cm) equilibrated with 50 mM Tris-HCl buffer allowing the adsorption of a part of cytochrome c_3 present in the wash fraction. Then, the hydrogenase was adsorbed on a DE-52 column (4.3 x 21 cm) equilibrated with 20 mM Tris-HCl buffer. The column was washed with 150 ml of the same buffer and the enzyme was eluted by means of a non-linear gradient of Tris-HCl buffer (50-200 mM, total volume 600 ml). The active fraction (53 ml) was loaded on a sephacryl column (5 x 100) equilibrated with 20 mM Tris-HCl buffer and most of the brown band of hydrogenase was separated from the c_3 type cytochrome at this step. The subsequent active fractions were pooled and adsorbed onto a hydroxylapatite (Bio-Gel HTP) column (2.5 x 9 cm) equilibrated with 10 mM Tris-HCl buffer. The elution which was achieved by a non-linear gradient of potassium phosphate buffer pH 7.6 (5-150 mM, total volume 350 ml) allowed the complete separation of the hydrogenase from the slight amount of c_3 type cytochrome still present in the preparation. The last step of this purification procedure includes a second filtration on a Sephacryl column. The specific activity of the pure enzyme reached 90 μmoles H_2 evolved/min./mg protein. The purification of D. gigas hydrogenase is summarized in Table I.

TABLE 1

Purification scheme of hydrogenase from Desulfovibrio gigas

STEP	VOLUME (ml)	PROTEIN (ml)	TOTAL UNITS*	SPECIFIC ACTIVITY	RECOVERY (%)
Wash fraction	270	36	18,460	1.9	100
After 1st DEAE	250	19	17,500	3.7	94.8
After Silica gel	250	18	14,500	3.2	78.5
After 2nd DEAE	53	27.5	13,356	9.2	72.3
After 1st Sephacryl	94	4.45	9,772	23.4	52.9
After hydroxylapatite	53	2.6	9,116	66.2	49.4
After 2nd Sephacryl	74	1.3	8,732	90.8	47.3

*One unit = 1 µmole hydrogen evolved per minute in the dithionite-methyl viologen assay

RESULTS

Purity of the enzyme. Polyacrylamide gel electrophoresis of the purified enzyme at pH 8.9 and the location of the hydrogenase activity on in the presence of methyl viologen and H_2 indicated that the preparation was homogeneous. Furthermore, when the purified enzyme was subjected to ultracentrifugation in 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl at 4° C, a single symmetrical boundary was observed.

Molecular weight. The molecular weight of hydrogenase determined by analytical ultracentrifugation at equilibrium sedimentation (17) was estimated to be 89,500 ± 1,880 and the partial specific volume of the enzyme has been found to be 0.73_4. The molecular weight of hydrogenase was also estimated from the molecular weight of its subunits obtained by SDS gel electrophoresis (18). This procedure revealed the presence of two different subunits : the molecular weight of the larger component was estimated to be 62,000 and that of the smaller component, 26,000. These results indicate that the native

enzyme exists as a dimer constituted by two different subunits which are not covalently bound since the dissociation occured also in the absence of 2-mercapto-ethanol. No hydrogenase activity was detected with the SDS gel electrophoresis.

Absorption spectrum. The UV-visible absorption spectrum of hydrogenase is presented on Fig. 1. In its oxidized state, it exhibits a broad absorption peak around 400 nm with a slight shoulder in the 325 nm region consistent with the non heme iron nature of the enzyme. The millimolar extinction coefficients of the purified enzyme at 400 and 280 nm were 46.5 and 170 respectively. Thus the ratio $A_{400\ nm}/A_{280\ nm}$ is 0.27.

Iron and acid-labile sulfide analysis. Analysis of the hydrogenase preparation for iron indicated the presence of 12 atoms per molecule (average of four estimations with different preparations). The amount of acid-labile sulfide was lower (9-10 atoms) when measured by the method of Fogo and Popowsky (19), however, the procedure of Suhara et al. (20) allowed to obtain values close to 12 atoms per molecule.

Extrusion of the Fe-S centers of hydrogenase. Quantitative extrusion of the Fe-S cores of the active site of hydrogenase has been accomplished with a benzenethiol/Fe molar ratio of 150:1. The results are presented on Fig. 2 The spectra of the extruded clusters (spectra 2 and 3) exhibiting a peak at 458 nm is consistent with the tetrameric nature of the Fe-S centers of hydrogenase. The final A_{458} value obtained after addition of ferricyanide was used to estimate the number of F_4S_4 centers of the enzyme (12). The results indicate that this preparation of hydrogenase contains 2.8 F_4S_4 clusters per molecule since the A_{458} value corresponds to 93 % of the value for complete extrusion based on 12 g-atom Fe/mole.

Amino-acid composition. The amino-acid composition of D. gigas hydrogenase is shown in Table 2 and compared with D. vulgaris , Miyazaki hydrogenase. The acidic properties of hydrogenase are explained by the preponderance of acidic amino-acids. The ratio Asx + Glx/Lys + Arg is about 2.9. It has a

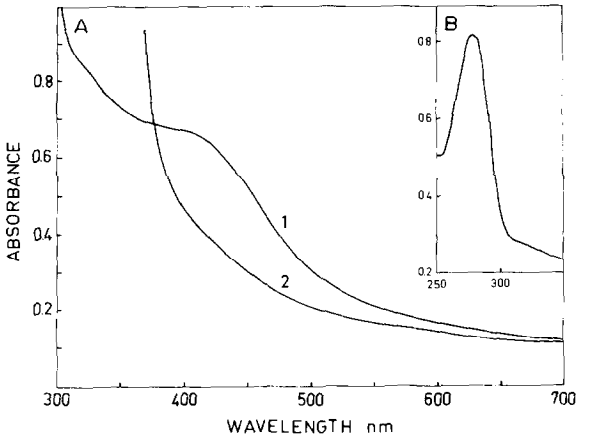

Figure 1 - Absorption spectra of D. gigas hydrogenase. A 1 cm light path cuvette was used. A - 1. The oxidized spectrum of the enzyme (under Ar) at 1.3 mg/ml in 20 mM Tris-HCl, pH 7.6 - 2. Solution 1 after reduction with slight excess of sodium dithionite. B - The UV absorption spectrum of the enzyme at 0.43 mg/ml in the same buffer.

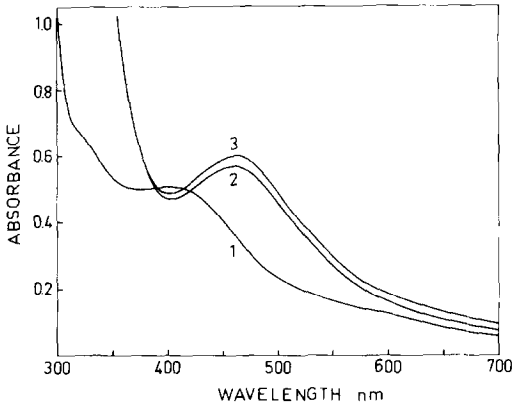

Figure 2 - Active site core extrusion of D. gigas hydrogenase in 4:1 v/v HMPA/H_2O (50 mM Tris-HCl, pH 8.5) under anaerobic conditions (l = 1 cm). 1) 2 ml of 12.7 µM hydrogenase ; 2) Solution 1 + 6 µl benzenethiol 10 min. after mixing ; 3) Solution 2 + 3 µl of 29 mM $K_3Fe(CN)_6$ 10 min. after mixing.

remarkably high content of aromatic residues and hydrophobic amino-acids as the hydrogenase from D. vulgaris, Miyazaki. It contains about 21 cysteine residues and 18 methionine residues for a total content of 876 residues which is in concordance with the measured molecular weight of 89,500.

TABLE 2

Amino acid composition of *Desulfovibrio* hydrogenases

Amino acid composition*	*Desulfovibrio gigas*	*Desulfovibrio vulgaris* Miyazaki[1]
Lysine	53	44
Histidine	26	24
Arginine	34	26
Tryptophane[a]	22	27
Aspartic acid	74	76
Threonine	47	49
Serine	28	37
Glutamic acid	93	69
Proline	47	57
Glycine	71	71
Alanine	75	85
Cystine (Half)[b]	21	19
Valine	100	62
Methionine[b]	18	11
Isoleucine	35	37
Leucine	70	54
Tyrosine	33	27
Phenylalanine	29	33
Total residues	876	808

*Moles per mole of protein to the nearest integer
[a]Determined spectrophotometrically according to Edelhoch (23)
[b]Cysteine and methionine were determined as cysteic acid and methionine sulfone after performate oxidation of the protein.
[1]From Yagi et al. (4).

DISCUSSION

The physico-chemical properties of the hydrogenase from D. gigas were compared to those of D. vulgaris, Miyazaki and C. pastorianum enzymes (Table 3). Most of the properties of Desulfovibrio species hydrogenases are similar, however, the proteins differ by their specific activity and the content of iron and labile sulfide. On the other hand, D. gigas enzyme contains an identical amount of F_4S_4 clusters as compared to C. pastorianum hydrogenase but differs from this latter by its specific activity, molecular weight, subunit structure and amino-acid composition. Furthermore, these enzymes show different specificity for the natural electron carriers in the hydrogen evolution or absorption reactions : hydrogenase from Desulfovibrio has been found to be specific to cytochrome c_3 (8, 25) whereas C. pastorianum enzyme is specific to ferredoxin (1). Although D. gigas and D. vulgaris hydrogenases exhibit similar specificity for cytochrome c_3, a different degree of dependency of its activity toward this natural electron carrier exists. Indeed, D. vulgaris hydrogenase activity in hydrogen evolution assay with methyl viologen is strongly stimulated in the presence of cytochrome c_3 (26) whereas we have observed that D. gigas enzyme activity is unchanged when cytochrome c_3, cytochrome cc_3 or ferredoxin are added. This could explain the difference observed in the specific activities of the two proteins since in the case of D. vulgaris, Miyazaki, hydrogenase activity is about 5-fold enhanced with cytochrome c_3 (26). Thus, the specific activities of the two enzymes are comparable when measured without natural electron carrier. In addition, in contrast to D. gigas hydrogenase properties, cytochrome c_3 is involved in the reduction of methyl viologen under H_2 and for the reduction of hydrogenase itself with a preparation of D. vulgaris hydrogenase (4, 26). This could be due to differences between cytochromes c_3 in relation to changes in redox properties of these hemo-proteins (27) which are characterized by extensive differences in their primary structures (28). The interest of the study of Desulfovibrio hydrogenase is in the fact that the catalytic properties of

TABLE 3

Physico-chemical properties of hydrogenases from Desulfovibrio and Clostridium

	Desulfovibrio gigas	Desulfovibrio[1] vulgaris	Clostridium[2] pastorianum
Specific activity	90.8	610	500
Molecular weight	89,500	89,000	60,500
Polypeptide chains	2	2	1
Iron (atoms/mole)	12	7-9	12
Acide-labile sulfide (moles/mole)	12	7-8	12
Half-Cystine	21	19	12
Number of F_4S_4 centers	3	(2)	3
Extinction coefficients (cm^{-1} mM^{-1} at 400 nm)	46.5	47	25.3[a]

[1]From Yagi et al. (4)
[2]From Chen and Mortenson (1) and Chen et al. (10)
[a]This value was determined at 400 nm with H_2-reduced hydrogenase (Chen and Mortenson, 1).

the protein i.e. the dihydrogen activation and its heterolytic cleavage (12) can be separated from its electron carrying properties toward ferredoxin and flavodoxin which involve the presence of cytochrome c_3 (25). One has to point out that C. pastorianum hydrogenase is able to transfer its electrons to ferredoxin when Desulfovibrio hydrogenases cannot, although the two enzymes have the same number of 4 Fe-4S clusters. It is to be expected that the catalytic properties of Desulfovibrio hydrogenase that can be followed by the HD exchange reaction, will be different in the presence or in the absence of cytochrome c_3.

Very recently a purification scheme for a hydrogenase from D. vulgaris strain Hildenborough has been published (29). Although this protein has

an iron and labile sulfide content similar to the D. gigas enzyme, its stability toward oxygen together with its extremely high specific activity seem to indicate that it represents a different type of hydrogenase.

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REFERENCES

1. Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta, 371, 283-98.
2. Le Gall, J., DerVartanian, D.V., Spilker, E., Lee, J.P. and Peck, H.D. Jr. (1971) Biochim. Biophys. Acta, 234, 525-30.
3. Gitlitz, P.H. and Krasna, A.I. (1975) Biochemistry, 14, 2561-67.
4. Yagi, T., Kimura, K., Daidoji, H., Sakai, F., Tamura, S. and Inokuchi, H. (1976) J. Biochem., 79, 661-71.
5. Schneider, K. and Schlegel, H.G. (1976) Biochim. Biophys. Acta, 452, 66-80.
6. Gogotov, I.N., Zorin, N.A. and Kondratieva, E.N. (1976) Biokhimiya, 41, 836-42.
7. Adams, M.W.W. and Hall, D.O. (1977) Biochem. Biophys. Res. Comm., 77, 730-737.
8. Yagi, T., Honya, M. and Tamiya, N. (1968) Biochim. Biophys. Acta, 153, 699-705.
9. Bell, G.R. (1973) In "Studies on electron transfer systems in Desulfovibrio : purification and characterization of hydrogenase, catalase and superoxyde dismutase. PH.D. Thesis.
10. Chen, J.S., Mortenson, L.E. and Palmer, G. (1976) In "Iron and Copper proteins", Advances in Experimental Medicine and Biology, 74, 68-82. Yasunobu, K.T., Mower, H.F. and Hayaishi, O., eds., Plenum Press, New York.
11. Bell, G.R., Lee, J.P. and Le Gall, J. (1973) Bacteriol. Proceed. P 319.
12. Gillum, W.O., Mortenson, L.E., Chen, J.S. and Holm, R.H. (1977) J. Am. Chem. Soc., 99, 584-95.
13. Nakos, G. and Mortenson, L.E. (1971) Biochim. Biophys. Acta, 227, 576-83.
14. Le Gall, J., Mazza, G. and Dragoni, N. (1965) Biochim. Biophys. Acta, 56, 385-87.
15. Peck, H.D. Jr. and Gest, H. (1956) J. Bacteriol., 71, 70-80.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-75.
17. Yphantis, D.A. (1964) Biochemistry, 3, 297-317.
18. Weber, K. and Osborn, M. (1969) J. Biol. Chem., 244, 4406-12.
19. Fogo, J.K. and Popowsky, M. (1949) Anal. Chem., 21, 732-34.
20. Suhara, K., Kanayama, K., Takemori, S. and Katagiri, M. (1974) Biochim. Biophys. Acta, 336, 309-17.

21. Moore, S. and Stein, W.H. (1963) Chromatographic determination of amino-acids by the use of automatic recording equipment, In Methods in Enzymology, S.P. Colowick and N.O. Kaplan (eds.) vol. 6, p. 819-31, Academic Press Inc., New York.
22. Hirs, C.H.W. (1967) Determination of cystine and cysteic acid, In Methods in Enzymology, C.H.W. Hirs (ed.) vol. 11, p. 59-62, Academic Press Inc., New York.
23. Edelhoch, H. (1967) Biochemistry, 6, 1948-54.
24. Bell, G.R., Le Gall, J. and Peck, H.D. Jr. (1974) J. Bacteriol., 120, 994-97.
25. Bell, G.R. and Le Gall, J. (unpublished results).
26. Yagi, T. (1970) J. Biochem., 68, 649-57.
27. Bruschi, M., Hatchikian, E.C., Golovleva, L.A. and Le Gall, J. (1977) J. Bacteriol., 129, 30-38.
28. Dobson, C.M., Hoyle, N.J., Geraldes, C.F., Wright, P.E., Williams, R.J.P., Bruschi, M. and Le Gall, J. (1974) Nature, 249, 425-29.
29. Van Der Westen, H.M., Mayhew, S.G. and Veeger, C. (1978) FEBS Letters, 86, 122-26.