BBA Report

BBA 41176

Evidence for the involvement of non-heme iron in the active site of hydrogenase from *Desulfovibrio vulgaris*

J. LEGALL, D.V. DERVARTANIAN, E. SPILKER, JIN-PO LEE and H.D. PECK, JR.
Department of Biochemistry, University of Georgia, Athens, Ga. 30601 (U.S.A.)
(Received April 13th, 1971)

SUMMARY

1. Hydrogenase (hydrogen:cytochrome c_3 oxidoreductase) of high specific activity has been purified to near homogeneity as judged by disc electrophoresis and in the analytical ultracentrifuge, and found to contain 3.5 iron and 3.2 labile-sulfide atoms per molecule (of mol. wt. 60 000).

2. Hydrogenase was found to exist in a dimer form of mol. wt. 60 000 and a monomer form of 30 000. The dimer–monomer forms were concentration-dependent with the dimer form favored at high protein concentrations.

3. Light absorption and EPR studies definitely indicate a redox functional iron-sulfide unit at the active site. On reduction with H_2, a $g = 1.86$ EPR absorption was observed. This is the Beinert "$g = 1.9$" type of EPR absorption observed in other iron-sulfide proteins such as plant and bacterial ferredoxins.

Hydrogenase from *Desulfovibrio vulgaris* (hydrogen:cytochrome c_3 oxidoreductase) has been characterized as exhibiting activity with the classical dyestuffs and cytochrome c_3 but not clostridial-type ferredoxin. This enzyme has been fractionated and studied by a number of investigators who have reported the presence of iron[1–4]. In contrast to the clostridial hydrogenase[5,6], hydrogen:ferredoxin oxidoreductase (EC 1.12.1.1), no evidence exists for a requirement of flavin or molybdenum for activity.

Conclusive studies on the nature of the iron involvement have been limited due to low yields of hydrogenase after purification and the extreme lability of the enzyme in the presence of oxygen. Haschke and Campbell[3] have recently purified hydrogenase from *D. vulgaris* and reported the enzyme contained non-heme iron although there was less than 1 iron and sulfide atom per mole enzyme and the absorbance at 408 nm was low. Nakos and Mortenson[1] recently isolated hydrogenase from *Clostridium pasteurianum* with 4 iron and 4 labile-sulfide atoms per molecule

of mol. wt. 60 000 and also reported that hydrogenase could exist in a monomer form of mol. wt. 30 000. No optical spectrum or evidence for the role of iron in enzymatic activity was presented.

In this paper we describe the isolation of hydrogenase from *D. vulgaris* of high specific activity containing a high and stoichiometric amount of iron and labile sulfide and possessing the absorption spectrum of a typical iron-sulfide protein. Absorption changes in the optical and EPR spectra in the presence of substrate, molecular hydrogen, indicate the involvement of non-heme iron in the active site of the hydrogenase.

The enzyme was extracted from 2 kg of *D. vulgaris*, strain Hildenborough, as previously described[7,8]. The first purification steps essentially removed cytochromes c_3 and c_{553} and have been published[7,8]. Most of the hydrogenase activity is retained on the calcinated alumina column together with cytochrome cc_3 [8]. The alumina column was eluted with 100 mM potassium phosphate buffer (all buffers used in the purification were kept at pH 7.6), containing 1 mM 2-mercaptoethanol, the eluates dialyzed overnight against 10 l of 10 mM Tris–HCl containing 1 mM 2-mercaptoethanol and after dialysis centrifuged to remove a slight precipitate, then adsorbed on a 4 cm x 50 cm DEAE-cellulose column. Cytochrome cc_3 was not adsorbed and was stored for later purification[8]. The adsorbed proteins were eluted with 100 mM Tris–HCl buffer, concentrated to approximately 120 ml (Diaflo ultrafilter, Amicon Corp.), passed through first a Sephadex G-75 column (5 cm x 100 cm) and then a Sephadex G-50 column of the same dimensions. The fractions containing hydrogenase activity were collected and adsorbed on DEAE-cellulose (4 cm x 22 cm). The proteins were eluted with a linear gradient concentration (100–600 mM Tris–HCl buffer). The first band contained the hydrogenase activity. The purification scheme is summarized in Table I.

TABLE I

PURIFICATION SCHEME OF HYDROGENASE

Enzyme activity was monitored with a manometric benzyl viologen assay. Assay conditions: side arm: enzyme, 0.02 ml; center well: 10% $CdSO_4$ solution, 0.05 ml; 10% NaOH, 0.05 ml; main-compartment: 100 mM potassium phosphate buffer; benzyl viologen, 2% solution, 0.40 ml; distilled water to 3.0 ml; temperature, 30°; atmosphere, H_2. The unit of activity was defined as μmoles H_2 oxidized per min. Protein was determined by the Biuret method[13]. When hydrogenase was present in Tris buffer, an appropriate blank was run.

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Total units*	*Specific activity (units/mg)*
Crude extract	2960	88.0	976 000	3.7
Alumina column eluate	320	27.5	107 000	12.8
After diaflo concentration	120	53.5	84 000	12.3
After Sephadex G-75/G-50	250	4.1	29 500	28.8
After DEAE-cellulose (2nd)	105	0.8	4 600	53.0*

*64 units/mg protein as defined by Haschke and Campbell[3] using methyl viologen as terminal electron acceptor and a molar absorptivity of 12 000 cm^{-1} at 600 nm for methyl viologen[14].

Hydrogenase was purified approx. 15-fold as compared to cell-free extracts. Our most purified preparation revealed a single major band as indicated by analytical disc electrophoresis on polyacrylamide gel. A small contaminant (about 3%) was also observed and attributed to a flavoprotein. Direct activity determination[9] on the gel revealed that enzymatic activity was associated only with the major band. Chemical analysis for iron by the method of Massey[10], for labile sulfide by the modification of Brumby *et al.*[11] and for molybdenum by the dithiol method[12] were performed. Our most purified preparation of hydrogenase was found to contain 58.6 nmoles of iron per mg protein (determined by the Biuret method[13]) and 53.1 nmoles labile sulfide per mg protein. Variable trace amounts of molybdenum (0.01–0.2 nmole Mo per mg protein) were occasionally detected. Sedimentation velocity measurements★ indicated that hydrogenase existed in dimer-monomer forms. At high protein concentrations (about 4 mg/ml) a single peak was observed with a mol. wt. of approx. 60 000 ($s_{20,w}$ = 4.49). At dilute concentrations (about 0.8 mg/ml a mixture of the dimer and monomer forms were detected with about 34% in the monomer form of approx. mol. wt. 30 000 and 66% in the dimer form of approx. mol. wt. 60 000 as determined by sedimentation equilibrium measurements using the Beckman optical scanning unit★.

The molecular weights of the dimer and monomer forms are very similar to those reported for hydrogenase from *C. pasteurianum*[1]. Based on a mol. wt. of 60 000 there are then 3.5 atoms iron and 3.2 atoms of labile sulfide per molecule. The lower values (instead of 4.0) are probably due to the presence of apo-protein resulting from loss of labile sulfide and iron during purification and/or lability of the hydrogenase.

The light-absorption spectrum of hydrogenase is presented in Fig. 1. A single maximum was found at 408 nm. The molar absorptivity per iron atom at 408 nm was 4430 cm^{-1}, in the range of similar iron-sulfide proteins[15,16]. The ratio of absorbance at 280 nm to 408 nm was 5.14 or 2.6-fold lower than that reported by Haschke and Campbell[3] Also in contrast to the latter study a higher iron and sulfide content and different molecular weight were found.

On reduction of hydrogenase with H_2 under anaerobic conditions a decrease of light absorption is observed but only after a lag period of about 7 min. At maximum reduction (40 min), the decline in absorption at 408 nm corresponds to 30% and that at 450 to 36%. The subsequent addition of the chemical reductant, sodium dithionite, results in onl a slight further decline of absorption.

Low-temperature EPR spectroscopy performed as previously described[17] reveals a small signal at $g = 2.02$ (Fig. 2,A) in the oxidized state of hydrogenase. Based on EPR parameters, *e.g.* g values and microwave power variation, this signal may be assigned to high-spin ferric iron in a weak crystal field (*cf.* ref. 18). On reduction with H_2 under anaerobic conditions for 1–2 min at 25° a small increase in the $g = 2.02$ signal was observed (Fig. 2,B). On further reaction with H_2 this signal declines and is replaced by a strong absorption at $g_\perp = 1.86$ and $g_\parallel = 2.03$ attributable to reduction of a binuclear form of iron sulfide (Fig. 2,C and D). This type of "$g = 1.9$" signal first observed by Beinert and Sands[19] has since been detected in a wide variety of iron-sulfide proteins containing a minimum two iron–two sulfide redox unit[1] The signal at $g = 1.86$ did not appear until at least 7–8 min and reached almost maximal intensity within 40 min (Fig. 2,D), in good agreement with concurrent light-absorption studi

★We are indebted to Mr. Terry Spencer for the molecular weight determinations.

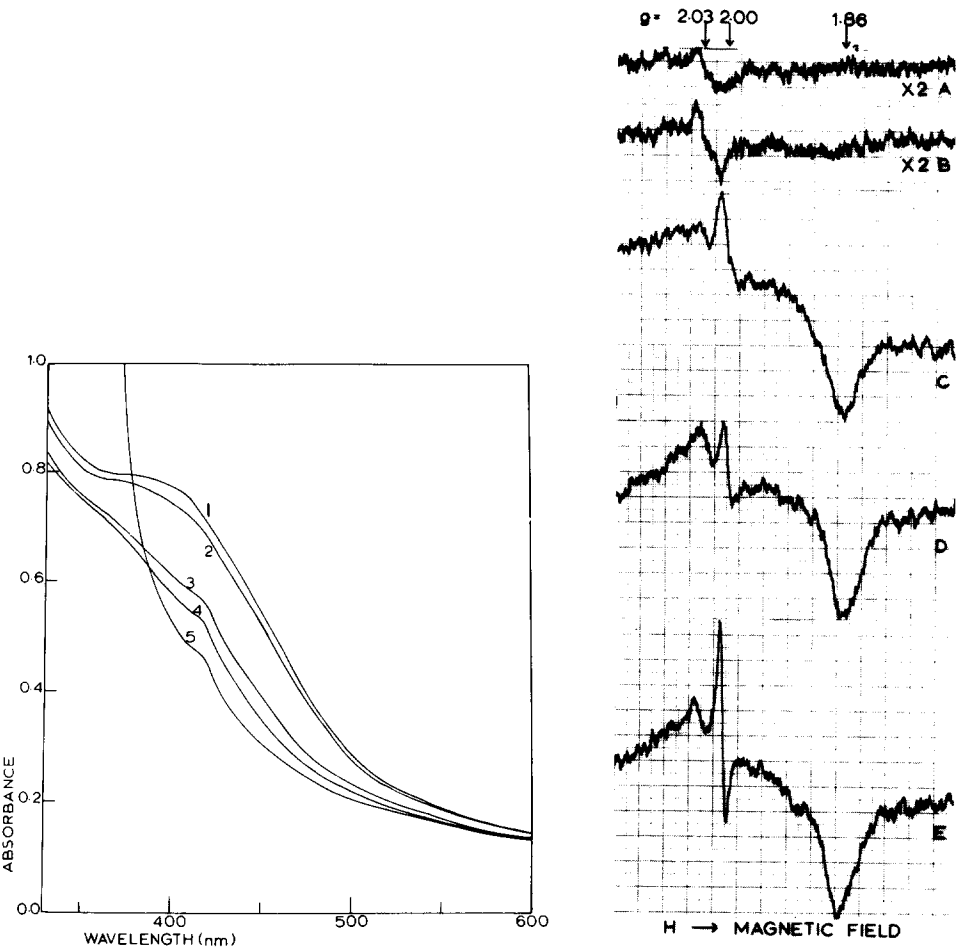

Fig. 1. Light-absorption spectra of hydrogenase (2.5 mg/ml) in 100 mM potassium phosphate buffer (pH 7.6), vol. 1 ml. Spectra were taken in a 1-ml anaerobic quartz Thunberg-type cuvette (Hellma) of 1 cm light path in a Cary Model 14 recording spectrophotometer at 25°. 1, oxidized (under He); 2, cuvette placed under H_2 atmosphere by vacuum-H_2 cycling, and spectra recorded at intervals, after 8 min; 3, after 19 min; 4, after 37 min; 5, after reduction with slight excess of sodium dithionite.

Fig. 2. EPR spectra of hydrogenase (13.2 mg/ml) in 100 mM potassium phosphate buffer (pH 7.6), in an EPR anaerobic quartz tube previously described[8]; EPR conditions: frequency, 9.243 GHz; temperature, −186°; microwave power, 40 mW; modulation amplitude, 5.9 gauss; scanning rate, 400 gauss/min. A, initial oxidized (under He); B, after vacuum-H_2 cycling and 1 min under H_2; C, under H_2 after 23 min; D, under H_2 after 42 min; E, after reduction with slight excess of sodium dithionite.

Reduction with sodium dithionite (Fig. 2,E) results in only a slight further increase in the $g = 1.86$ absorption. A trace radical signal at $g = 2.00$ appearing in Fig. 2 (C and D) after incubation in the presence of H_2 is intensified on dithionite reduction (Fig. 2,E) and may be attributed to the small amount of flavoprotein contaminant.

Unlike the "$g = 1.9$" type of resonances observed in other iron-sulfide proteins, the EPR resonance of reduced hydrogenase is unusual in terms of its temperature behavior. The signal at $g = 1.86$ is observed at -50° whereas with other iron-sulfide proteins, the "$g = 1.9$" absorption would be extensively broadened and undetectable at such a high temperature.

Kleiner and Burris[6] reported that their hydrogenase preparation from *C. pasteurianum* which showed no optical features or contained iron could be reactivated with permolybdate. In view of the variable trace amounts of molybdenum found in this preparation we have studied the effect of added permolybdate (conditions as described by Kleiner and Burris) on enzyme activity and EPR absorption. Our results show that the permolybdate-treated hydrogenase did not exhibit increased enzyme activity. The EPR absorption initially present and after reduction with H_2 was not altered. However, after redu with H_2 to obtain almost maximal signal intensity at $g = 1.86$, the permolybdate-treated hydrogenase on subsequent dithionite addition did reveal a strong Mo(V) absorption, indicating that had H_2 reduced enzymatically molybdenum, presumably present as Mo(VI), the EPR absorption of Mo(V) would have been detected without the addition of dithionite.

The lag in reactivity observed in the optical and EPR studies as well as enzyme assays for this preparation of hydrogenase and throughout the literature of the enzyme is not readily explained. Fisher *et al.*[20] attributed this phenomenon to a reversible binding of oxygen with hydrogenase with the lag period due to the time required for deoxygenation as in the presence of the substrate, H_2. Such an explanation remains a possibility. The possible role of the dimer–monomer forms of hydrogenase in the lag phase requires further studies. It may be noted that the optical and EPR studies were done with relatively concentrated solutions of hydrogenase, conditions which favor the dimer form. However, enzyme assays are performed at much more dilute concentrations where the monomer form is presumably predominant.

The experiments described clearly establish the role of non-heme iron in the mechanism of action of hydrogenase from *D. vulgaris*. Yagi *et al.*[21] proposed differentiation of the hydrogenases from Clostridia and sulfate-reducing bacteria on the basis of electron acceptor specificity. Their study can be criticized because they did not test ferredoxin from the sulfate-reducing bacteria. In addition the hydrogenases from both types of bacteria appear to be similar in cofactor contents and molecular weights of dimer and monomer forms.

This research was supported by Research Grant GB-13242 awarded by the National Science Foundation to D.V.D.

REFERENCES

1 G. Nakos and L. Mortenson, *Biochim. Biophys. Acta*, 227 (1971) 576.
2 T. Yagi, *J. Biochem. Tokyo*, 68 (1970) 649.
3 R.H. Haschke and L.L. Campbell, *J. Bacteriol.*, 105 (1971) 249.
4 J.C. Sadana and D. Rittenberg, *Arch. Biochem. Biophys.*, 108 (1964) 255.
5 A.L. Shug, P.W. Wilson, D.E. Green and H.R. Mahler, *J. Am. Chem. Soc.*, 76 (1954) 3355.
6 D. Kleiner and R.H. Burris, *Biochim. Biophys. Acta*, 212 (1970) 417.
7 J. LeGall and M. Bruschi-Heriaud, in K. Okumuki, M.D. Kamen and I. Sekuso, *Structure and Function of Cytochromes*, Univ. of Tokyo Press, 1968, p. 467.
8 J. LeGall, M. Bruschi-Heriaud and D.V. DerVartanian, *Biochim. Biophys. Acta*, 234 (1971) 499.
9 B.A.C. Ackrell, R.N. Asato and H.F. Mower, *J. Bacteriol.*, 92 (1966) 828.
10 V. Massey, *J. Biol. Chem.*, 229 (1957) 763.

11 P.E. Brumby, R.W. Miller and V. Massey, *J. Biol. Chem.*, 240 (1965) 1451.
12 L.J. Clark and J.H. Axley, *Anal. Chem.*, 27 (1955) 2000.
13 A.G. Cornall, C.J. Bardawill and M.M. David, *J. Biol. Chem.*, 177 (1949) 751.
14 P.A. Trudinger, *Anal. Biochem.*, 36 (1970) 222.
15 D.V. DerVartanian, Y.I. Shethna and H. Beinert, *Biochim. Biophys. Acta*, 194 (1969) 548.
16 H. Komai, V. Massey and G. Palmer, *J. Biol. Chem.*, 244 (1969) 1692.
17 D.V. DerVartanian and R. Bramlett, *Biochim. Biophys. Acta*, 220 (1970) 443.
18 G. Palmer, R.H. Sands and L.E. Mortenson, *Biochem. Biophys. Res. Commun.*, 23 (1966) 357.
19 H. Beinert and R.H. Sands, *Biochem. Biophys. Res. Commun.*, 3 (1960) 41.
20 H.F. Fisher, A.I. Krasna and D.R. Henberg, *J. Biol. Chem.*, 209 (1954) 569.
21 T. Yagi, M. Honya and N. Tamiya, *Biochim. Biophys. Acta*, 153 (1968) 699.