Biochimica et Biophysica Acta, 681 (1982) 330–334
Elsevier Biomedical Press

BBA Report

BBA 40010

ON THE ACTIVE SITE OF HYDROGENASE FROM *CHROMATIUM VINOSUM*

S.P.J. ALBRACHT, K.J. ALBRECHT-ELLMER, D.J.M. SCHMEDDING and E.C. SLATER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

(Received February 15th, 1982)

Key words: Hydrogenase; ESR; Nickel; Iron-sulfur protein; Active site; (C. vinosum)

Isotope substitution of ^{57}Fe ($I = 1/2$) for ^{56}Fe has a pronounced effect on the two EPR signals of hydrogenase of *Chromatium vinosum*. It is proposed that signal 1, the intensity of which is increased several-fold by a deoxygenation-oxygenation cycle with a simultaneous increase of a signal from Fe^{3+}, is due to a [3Fe-xS] cluster. It is further proposed that signal 2 is caused by a magnetic interaction of a $[4Fe\text{-}4S]^{3+}$ cluster with an unidentified paramagnet. The addition of 10 μM Ni to the culture medium (already containing 1 μM Ni) increased the enzyme activity 3–6-fold, without effect on the growth of the bacterium. Addition of ^{61}Ni ($I = 3/2$) to the medium did not change the EPR spectrum of hydrogenase. From a comparison of the EPR signal intensities and the enzyme activities it is concluded that, in the hydrogenase preparation as isolated, molecules containing a [3Fe-xS) cluster are not active, and that active molecules have a $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster plus an as yet unidentified paramagnetic redox component. The latter is thought to be the primary site of interaction of the enzyme with H_2. Ni is considered as a possible candidate for this component.

At present three classes of hydrogenase (hydrogen:(acceptor) oxidoreductase, EC 1.12.-.-) are known [1–3]: (i) enzymes containing four Fe and four acid-labile S atoms per molecule, presumably arranged in a single [4Fe-4S] cluster; (ii) enzymes containing 12 Fe and 12 S atoms, arranged in three [4Fe-4S] clusters; (iii): enzymes with 12 Fe and 12 S atoms as well as flavin, but where, in contrast to the second class, two 2Fe and two 4Fe clusters seem to be present [4]. Hydrogenase from *Chromatium vinosum* belongs to the first class. The EPR spectrum of the oxidized enzyme has been considered as indicative for a $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster [5,6]. A previous report from this laboratory [7] demonstrated the presence of a second signal that does not behave like an $S = 1/2$ signal. In addition, it was observed that the reduced enzyme can transfer two reducing equivalents to ferricytochrome *c*, suggesting the presence of a second redox component in the enzyme [7].

In this report we have further characterized the EPR signals of the enzyme and describe the relation of these signals to the enzymic activity.

C. vinosum, strain DSM 185, was grown as described by Hendley [8] with $Na_2S_2O_3$ as electron donor. Growth on ^{61}Ni ($I = 3/2$, final concentration 10 μM, final enrichment 80%) and ^{57}Fe ($I = 1/2$, final concentration 15 μM, final enrichment 92%) was carried out in 20-l bottles. Both isotopes were obtained from Rohstoff-Einfuhr GmbH, F.R.G. Hydrogenase was purified, by a modification of the previously described method [7], to a stage where the EPR spectrum in the *g* 2 region consisted only of signals due to the enzyme. Metal concentrations were determined by atomic absorption spectrometry, working either with a flame (Perkin Elmer 305) or with a graphite furnace (Perkin Elmer 560). EPR measurements were performed on a Varian E-9 EPR spectrometer as before [7]. Enzyme activity was measured ampero-

metrically [9] at 30°C in 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA with either 2 mM methyl viologen plus 50 mM $Na_2S_2O_4$ (H_2 production), or 5 mM benzyl viologen (H_2 uptake) in the presence of glucose (80 mM) plus glucose oxidase (0.4 mg/ml) to remove oxygen.

It was recently proposed [10] that Ni might be a constituent of hydrogenase in 'Knallgas' bacteria. The soluble hydrogenase from *Methanobacterium thermoautotrophicum* contains one Ni atom per molecule [11]. Since Ni ions can be paramagnetic in all their valency states and since we have previously found [7] that 75% of the Fe-S clusters in hydrogenase of *Chromatium* are EPR undetectable in the enzyme as isolated, possibly by magnetic interaction with another paramagnet, Ni is an attractive candidate for the second redox group in the enzyme. We have observed that when *Chromatium* is cultured in a mineral medium, containing 1 μM Ni as contaminant, further addition of Ni (10 μM) has no effect on the growth, but increases the hydrogenase activity 3–4-fold. Also, no extra Ni is needed for growth under H_2 and in this case Ni addition raises the hydrogenase contents of the cells 5–6-fold. This suggests that Ni is involved in the synthesis of the enzyme. Similar results have recently been reported for *Rhodopseudomonas capsulata* [12].

In Fig. 1, EPR spectra of hydrogenase from *Chromatium* cultured in media containing either ^{57}Fe, ^{61}Ni or natural isotopes can be compared. As seen in trace B, ^{61}Ni has no apparent effect on the line width of the two signals in the spectrum. In contrast, ^{57}Fe has a marked effect (trace C). The effect of ^{57}Fe on signals 1 and 2 is displayed in Figs. 2 and 3, respectively. The effect of isotope substitution of ^{57}Fe for ^{56}Fe (Fig. 2) proves for the first time that signal 1 is indeed due to an Fe-containing paramagnet. One nucleus gives rise to a hyperfine splitting in the *x-y* region (1.5 mT) that is exceptionally large for an Fe-S cluster [13]. The increase in apparent line width reflects weak interaction with one or more additional Fe nuclei. The EPR properties of the signal are those of a [4Fe-4S]$^{3+}$ or a [3Fe-*x*S] cluster [14]. The second signal (Fig. 3) also shows a clear broadening, proving the involvement of Fe. This strengthens one of the possibilities mentioned previously [7], namely that the second signal arises from a magnetic interaction between an Fe-S cluster and another paramagnet. Although Ni(III) would be a component very well suited as the missing paramagnet, the lack of detectable hyperfine splitting of the $I = 3/2$ nucleus (Fig. 1) does not allow any conclusion on the possible involvement of Ni in signal 2.

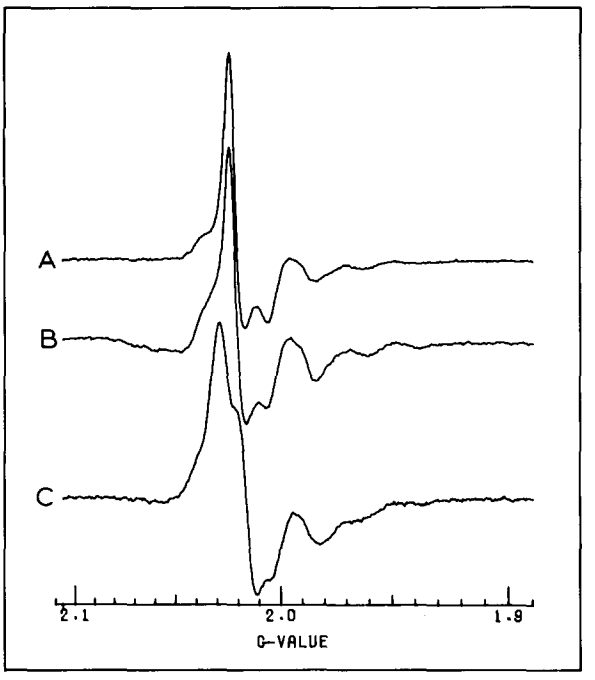

Fig. 1. EPR spectra of hydrogenase from *Chromatium* cells grown in media containing different isotopes. (A) Natural abundance (15 μM Fe and 1.2 μM Ni). (B) ^{61}Ni (15 μM Fe and 10 μM Ni, 80% enriched in ^{61}Ni). (C) ^{57}Fe (15 μM Fe, 92% enriched in ^{57}Fe, and 1.2 μM Ni). The specific H_2 production activities of the preparations used for A, B and C were 3.4, 1.2 and 0.3 μmol/min per mg, respectively. The spectra are presented with the same amplitude. EPR conditions: microwave frequency, 9256.3 MHz; temperature, 13 K; microwave power, 2.2 mW; modulation amplitude, 0.63 mT.

We have previously reported that the second signal disappears on removal of oxygen from the enzyme preparation and that, on subsequent contact with air, signal 1, that is not changed during deoxygenation, increases to an intensity equal to one spin per molecule. We have now found that a signal at *g* 4.3 increases in intensity concomitantly with the Fe-S signal (Fig. 4). Since conversion of 4 Fe clusters to 3 Fe clusters has recently been reported to take place under oxidizing conditions [15] and since a *g* 4.3 signal is given by Fe^{3+}

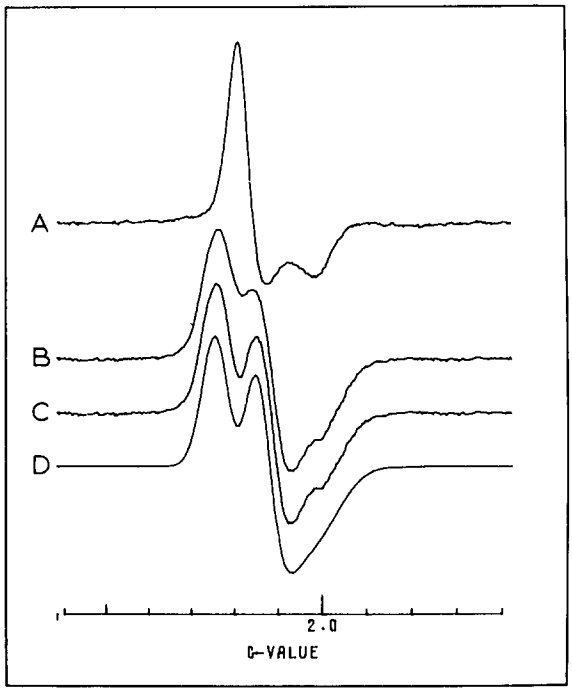

Fig. 2. Effect of ^{57}Fe on the line shape of signal 1. (A) EPR line shape of a normal enzyme after contact with 2 mM 2-mercaptoethanol for 4 h at 0°C. (B) Line shape of an enzyme containing 92% ^{57}Fe after the same treatment. The shoulder at g 2.00 is caused by a small interfering radical signal. (C) Line shape of an enzyme that would contain 100% ^{57}Fe. This was constructed as a difference B minus ($f \times$A), such that the signal intensity of ($f \times$A) was 8% of that of B. EPR conditions as in Fig. 1. (D) Simulation of C. The parameters to fit trace A ($g(x,y,z)$ 2.0183, 2.01639, 2.001 and width (x,y,z)=0.68, 1.1, 1.05 mT) were identical to those reported earlier [7] except for the $g(z)$ value, which was 0.0009 greater in Ref. 7. Trace C was fitted with these g values and width (x,y,z)=1.0, 1.2, 1.9 mT and a $I=1/2$ splitting with A (x,y,z)=1.5, 1.5, 2.0 mT. Spectra are presented with the same amplitude. The g value scale is in divisions of 0.01.

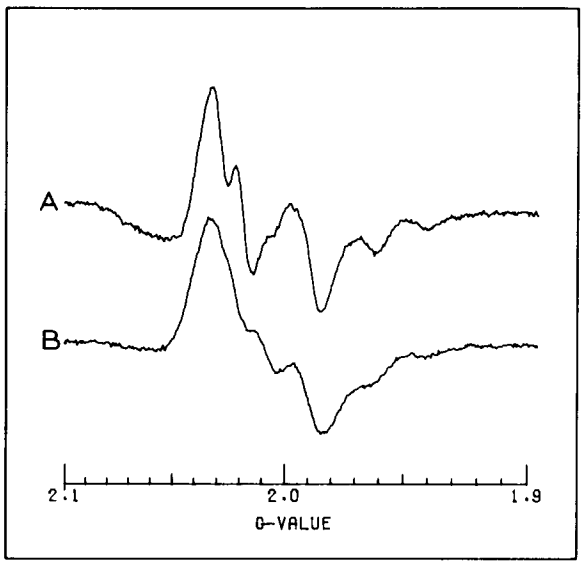

Fig. 3. Effect of ^{57}Fe on the line shape of signal 2. (A) EPR line shape of a normal enzyme. (B) Enzyme containing 92% ^{57}Fe. The traces are difference spectra obtained via subtraction of line shapes, as in Fig. 2, from those displayed in Fig. 1. EPR conditions: microwave frequency, 9257.9 MHz; temperature, 13 K; microwave power, 2.2 mW; modulation amplitude, 1.25 mT. Spectra are presented with the same amplitude.

($S=5/2$, rhombic environment), we suggest that the appearance of the two signals (Fig. 4) reflects such a conversion and that signal 1 is due to a [3Fe-xS] cluster. A corollary of this conclusion is that most of the enzyme molecules contain a [4Fe-4S] cluster that as such cannot be detected in EPR spectra of either the oxidized or the reduced enzyme.

We also noticed that the extent of the increase of signal 1 after the deoxygenation-oxygenation cycle as in Fig. 4 is preparation dependent and that the enzymatic activity is not related to the intensity of signal 1 in the enzyme as isolated, but rather to the concentration of the EPR-undetectable [4Fe-4S] cluster (Table I). Thus, protein molecules exhibiting the [3Fe-xS] signal seem to be enzymatically inactive. Although we consider it highly unlikely that the proposed [4Fe-4S] cluster in catalytically active molecules can, on its own, function as the active site and would be capable of taking up two reducing equivalents, these questions remain to be answered.

Very recently, experimental proof has been presented that Ni in the hydrogenase from *M. thermoautotrophicum* is reduced from Ni(III) to Ni(II) by H_2 [16]. In this case no EPR signals due to Fe-S clusters could be detected under any conditions. This indicate that the presence of an Fe-S cluster is not necessarily essential for hydrogenase activity.

CO is a competitive inhibitor of hydrogenase [3], but has no effect on the EPR spectrum of the *Chromatium* enzyme [6,7]. Also, NO has been reported to inhibit hydrogenase activity [17]. We

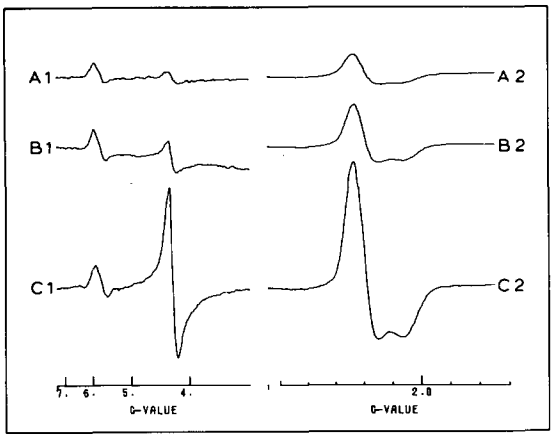

Fig. 4. Effect of a deoxygenation-oxygenation cycle on the EPR spectrum of hydrogenase. (A) Hydrogenase (preparation No. 3 from Table I) was six times evacuated and gassed with He. After 18 h at 4°C under He, a spectrum was recorded. (B) After thawing, the preparation was six times evacuated and gassed with air. After 1 h at 20°C under air a spectrum was recorded. (C) Spectrum after thawing and a further 1.5 h at 20°C. There was no further change in the signal amplitudes on additional contact with air for 1 h. The line at g 6 represents contaminating high-spin heme. EPR conditions: microwave frequency, 9259 MHz; temperature, 13 K; microwave power, 71 mW for the g 4 region and 2.2 mW for the g 2 region; modulation amplitude, 1.25 mT. The traces A1–C1 are displayed at a 5.6-times higher gain than the traces A2–C2.

found that the paramagnetic NO destroys the Fe-S cluster and forms an Fe^{2+}-NO compound ($S = 3/2$) with a characteristic signal with $g(x,y)$ 4 and $g(z)$ 2 as described earlier with other systems [18]. No signals were observed pointing to a possible reaction with Ni.

Summarizing, we propose that the active enzyme molecules as isolated contain a $[4Fe\text{-}4S]^{3+}$ cluster that magnetically interacts with another spin non-integer redox component, giving rise to signal 2. Oxygen removal apparently affects the spin state of this system and renders it EPR undetectable. The additional redox component is considered to be responsible for the primary reaction with H_2. Although our preparation always contain Ni (Table I) and under certain conditions show a very small EPR signal similar to that from hydrogenase in *Methanobacterium* [16], further investigations are needed to obtain direct experimental evidence for a possible involvement of Ni in the active cycle of the enzyme from *C. vinosum*.

We thank Dr. R.K. Thauer for drawing our attention to the possible role of Ni, for stimulating discussions and for sending us a manuscript prior to publication. We also thank Dr. J. Kragten and Mr. R. van Bennekom for their kind help with the Ni analysis. Part of this research was supported by the Netherlands Organization for the Advance-

TABLE I

HYDROGEN PRODUCTION BY HYDROGENASE IN RELATION TO THE STATE AND AMOUNT OF THE Fe-S CLUSTER

Preparation	Specific activity (μmol/min per mg)	[3Fe-xS] contents (%) [a]	Turnover number ($\times 10^{-3}$) mol H_2/mol cluster per min calculated on basis of			Ni per [4Fe-4S] (mol/mol)
			[3Fe-xS]	[3Fe-xS] after deoxygenation-oxygenation [b]	[4Fe-4S] [c]	
1	0.3	53	1.8	0.9	2.0	7.0
2	0.4	36	6.0	2.2	3.4	9.0
3	1.2	14	24.0	3.4	4.0	3.0
4 [d]	42.5	25	10.0	25	3.4	–

[a] Intensity of the EPR signal in the enzyme as isolated, relative to that of the enzyme after a deoxygenation-oxygenation cycle.
[b] Cluster concentration computed from the maximal EPR signal intensity after a deoxygenation-oxygenation cycle as in Fig. 4.
[c] Amount of EPR-undetectable [4Fe-4S] cluster that can be converted to a [3Fe-xS] cluster by deoxygenation-oxygenation.
[d] Calculated from data of Ref. 7 for the pure enzyme.

ment of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Krasna, A.I. (1979) Enzyme Microb. Techn. 1, 165–172
2 Weaver, P.F., Lien, S. and Seibert, M. (1980) Sol. Energy 24, 3–45
3 Adams, M.W.W., Mortenson, L.E. and Chen, J.-S. (1980) Biochim. Biophys. Acta 594, 105–176
4 Schneider, K., Cammack, R., Schlegel, H.G. and Hall, D.O. (1979) Biochim. Biophys. Acta 578, 445–461
5 Gitlitz, P.H. and Krasna, A.I. (1975) Biochemistry 14, 2561–2568
6 Strekas, T., Antanaitis, B.C. and Krasna, A.I. (1980) Biochim. Biophys. Acta 616, 1–9
7 Van Heerikhuizen, H., Albracht, S.P.J., Slater, E.C. and Van Rheenen, P.S. (1981) Biochim. Biophys. Acta 657, 26–39
8 Hendley, D.D. (1955) J. Bacteriol. 70, 625–634
9 Wang, R., Healey, F.P. and Myers, J. (1971) Plant Physiol. 48, 108–110
10 Thauer, R.K., Diekert, G. and Schönheit, P. (1980) Trends Biochem. Sci. 5, 304–306
11 Graf, E.-G. and Thauer, R.K. (1981) FEBS Lett. 136, 165–169
12 Takakuwa, S. and Wall, J.D. (1981) FEMS Microbiol. Lett. 12, 359–363
13 Anderson, R.E., Anger, G., Petersson, L., Ehrenberg, A., Cammack, R., Hall, D.O., Mullinger, R. and Rao, K.K. (1975) Biochim. Biophys. Acta 376, 63–71
14 Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, J., Orme-Johnson, W.H. and Münck, E. (1980) J. Biol. Chem. 255, 1793–1796
15 Thomson, A.J., Robinson, A.E., Johnson, M.K., Cammack, R., Rao, K.K. and Hall, D.O. (1981) Biochim. Biophys. Acta 637, 423–432
16 Albracht, S.P.J., Graf, E.-G. and Thauer, R.K. (1982) FEBS Lett. 140, 311–313
17 Krasna, A.I. and Rittenberg, D. (1954) Proc. Natl. Acad. Sci. U.S.A. 40, 225–227
18 Rich, P.R., Salerno, J.C., Leigh, J.S. and Bonner, W.D., Jr. (1978) FEBS Lett. 93, 323–326