THE EPR PROPERTIES OF NICKEL IN HYDROGENASE FROM *METHANOBACTERIUM THERMOAUTOTROPHICUM*

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1. Introduction

Nickel is required for the biosynthesis of hydrogenase in 'Knallgas' bacteria [1], *Methanobacterium thermoautotrophicum* [2], *Rhodopseudomonas capsulata* [3] and *Chromatium vinosum* [4]. The partially purified hydrogenase from *Methanobacterium* contains close to 1 Ni atom/molecule [2]. Nickel ions can be paramagnetic in all their valency states. An EPR signal is expected for the spin non-integer Ni(III)-ion and has been reported for Ni complexed with EDTA [5] or with peptides [6,7]. Membrane preparations of *Methanobacterium bryantii* also show an EPR signal that is possibly due to Ni [8]. We are not aware of reports on EPR spectra of purified Ni-containing enzymes. Here, we report on the EPR spectrum of hydrogenase from *Methanobacterium thermoautotrophicum* and prove, by isotope substition, that the spectrum is due to Ni.

2. Materials and methods

Methanobacterium thermoautotrophicum was grown as in [9]. For the isotope substition experiment cells were grown in a medium to which 10 μM ^{61}Ni was added. The isotope (enrichment 89.4%) was obtained from Rohstoff-Einfuhr GmbH. Hydrogenase was partially purified as in [2]. A further purification was achieved by gradient centrifugation (7.5–25% sucrose) for 5 h at 206 000 × g in a Beckmann VTi 50 rotor. The peak fraction (spec. hydrogen uptake act. 300 μmol . min^{-1} . mg protein^{-1} in the natural Ni-containing enzyme and 480 μmol . min^{-1} . mg protein^{-1} in the ^{61}Ni-containing enzyme) was concentrated by ultrafiltration and stored in liquid nitrogen.

Recording, processing, simulation and quantification of EPR spectra were done as in [10,11].

3. Results

The purified enzyme was searched for EPR signals over 7–80 K. Two signals were seen:

(i) A set of three lines at g = 2.3, 2.2 and 2.0, which were easily detectable up to 80 K;

(ii) A small signal at g = 4.3.

Signal (ii) is present in many proteins and mostly stems from Fe^{3+} (S = 5/2, rhombic ligand field) that is aspecifically bound. The signal in the g = 2.3–2.0 region, recorded under optimal conditions, is shown in fig.1A. When the enzyme is in contact with H_2 this signal disappears completely (fig.1B). The radical signal at g = 2.0 is due to benzylviologen which was added to enable visual inspection of the reduction process before freezing the sample. With another preparation the signal likewise disappeared in the absence of benzylviologen, and in that case the signal at g = 2 was absent. No other signals could be detected. When an aerobic solution containing excess $K_3Fe(CN)_6$ was subsequently added, 2/3rd of the intensity of the g = 2.3–2.0 signal was regained.

Fig.2 illustrates that the signal can be simulated as an effective S = 1/2 system. The intensity of the signal, measured directly via the area of the high-field peak or indirectly using the simulated line shape, corresponds to a spin concentration of 9.6 μM. The hydrogen uptake activity of the enzyme preparation was 530 μmol . min^{-1} . ml^{-1}. Assuming spec. act. 720 μmol . min^{-1} mg^{-1} for the pure enzyme [2] and M_r 60 000 [12], this corresponds to ~12.2 μM enzyme. The EPR signal thus is not due to an impurity.

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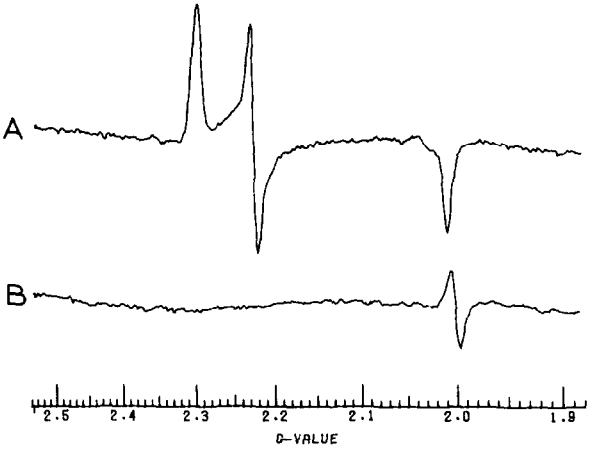

Fig.1. EPR spectra of hydrogenase from *Methanobacterium thermoautotrophicum:* (A) enzyme as isolated (spec. H_2 uptake act. 300 μmol . min^{-1} . mg^{-1}): (B) the enzyme solution was diluted 2-fold with 50 mM Tris–HCl (pH 8.0) and 50 μM benzylviologen was added. The mixture was then 6 times evacuated and gassed with H_2. After 2.5 h at 4°C under H_2 the sample was frozen in liquid nitrogen. EPR conditions: microwave frequency (*F*), 9240.4 MHz; temperature (*T*), 70 K; microwave power (*P*), 2.2 mW; modulation amplitude (*MA*), 1.25 mT; scanning rate (*SR*), 25 mT/min. The modulation frequency for these and other spectra in this report was 100 kHz.

The *g*-values, as well as the temperature dependence of the signal in fig.2, are very similar to those found for Ni in artificial complexes [5–7]. Since, however, the *g*-values of transition-metal ions are greatly determined by the ligand field, these values cannot be relied

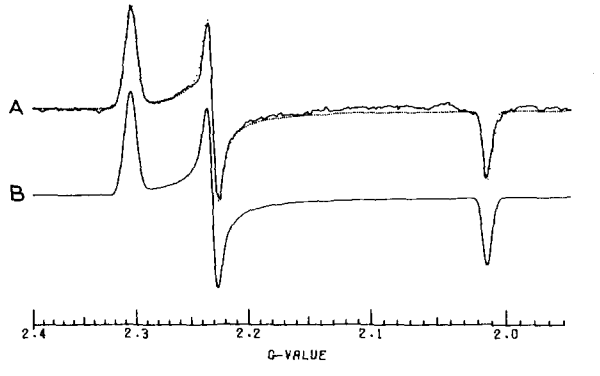

Fig.2. Simulation of the EPR spectrum of hydrogenase as a single $S = 1/2$ component. (A, solid line) Experimental spectrum. EPR conditions: *F*, 9239.4 MHz; *T*, 73 K; *P*, 2.2 mW; *MA*, 0.63 mT; *SR*, 6.25 mT/min. (B and A, dotted line) Simulation as a $S = 1/2$ system, using a Gaussian line-shape function and the following parameters: $g(x,y,z) = 2.3052, 2.231, 2.01471$ and widths (x,y,z) 1.4, 1.08, 1.1 mT.

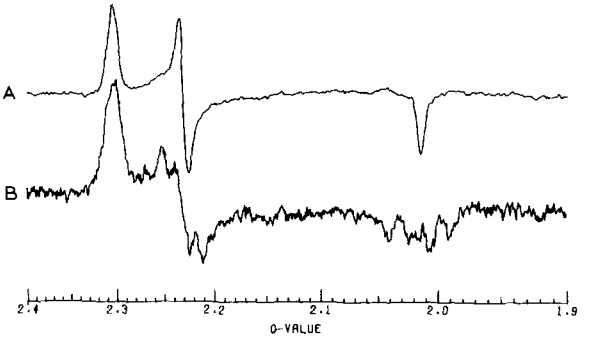

Fig.3. Comparison of the EPR spectra of hydrogenase from *Methanobacterium* grown on different Ni isotope mixtures: (A) growth was performed with natural Ni (natural abundance of ^{61}Ni is 1.19%); (B) growth in the presence of ^{61}Ni. The specific activity of the enzyme used for trace B was 480 μmol . min^{-1} . mg^{-1}. EPR conditions for (A) were as in fig.2. EPR conditions for (B) were the same as for (A) except for a scanning rate of 50 mT/min; the spectrum is an average of 30 scans and corrected for the base line recorded under identical conditions.

on for identification of the ion. We therefore also studied hydrogenase purified from cells grown on a medium enriched in ^{61}Ni ($I = 3/2$). The resultant EPR spectrum is compared with that of the normal enzyme (natural abundance for ^{61}Ni is 1.19%) in fig.3. This comparison proves that the signal is due to Ni. Especially the g_z line at $g = 2.01$ is clearly split into 4 lines. The presence of other Ni nuclei, none of which have a nuclear spin, in the preparation disturbs the hyperfine pattern in both the g_y and the g_z lines as demonstrated by the simulations in fig.4. In fig.4C the simulation of fig.2 was repeated but now supplemented with the hyperfine values ($A(x,y,z) = 0.75$, 1.5 and 2.71 mT) estimated from the experimental ^{61}Ni spectrum. This simulation cannot be correct since the isotope used was only 89.4% enriched in ^{61}Ni. In the growth medium this enrichment has further decreased and so the experimental spectrum is a mixture due to ^{61}Ni ($I = 3/2$) and the other natural isotopes ($I = 0$). This explains the difference between the simulation on basis of 100% enrichment (fig.4C) and the experimental spectrum (fig.4A). A rather good fit was obtained by assuming an enrichment of 80% (fig.4B). In this case, the intensity of the signal (fig.4A) estimated by double integration of the simulation (fig.4B), correlated to a spin concentration of 5.7 μM. Since the enzyme activity was ~440 μmol . min^{-1} . ml^{-1}, this corresponds to 0.6 mol Ni/mol enzyme.

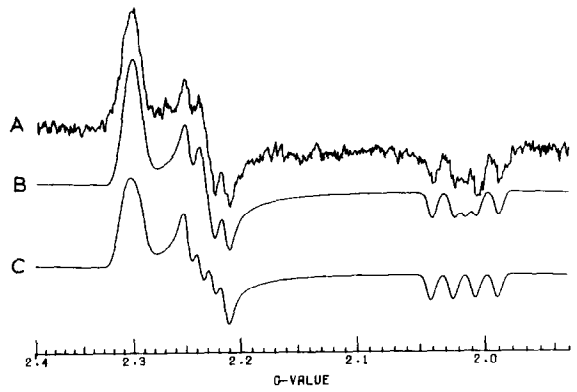

Fig.4. Analysis of the EPR spectrum of ^{61}Ni-containing hydrogenase. (A) Experimental spectrum as in fig.3B (B) simulation of the spectrum on basis of a ^{61}Ni enrichment of 80%, i.e., the signal is a composite (on basis of the double integral value) of 80% of the ^{61}Ni-signal simulation (C) and 20% of a simulation of the normal Ni-signal (fig.2B). (C) Simulation on basis of a ^{61}Ni enrichment of 100%. The hyperfine splitting values used for the simulation were $A(x,y,z)$ = 0.75, 1.5, 2.71 mT. The g-values used for (B,C) differ slightly from those used for fig.2B and were $g(x,y,z)$ = 2.3036, 2.2323, 2.0181; the line widths were as in fig.2B.

4. Discussion

The most surprising result of this EPR study on the hydrogenase from *Methanobacterium thermoautotrophicum* is the absence of any signal of an Fe–S cluster. All purified hydrogenases thus far reported [13,14] contain one or more Fe–S clusters, that show EPR signals in either the reduced or the oxidized state. Our enzyme, however, did not show such signals, neither in the isolated state, nor in the H_2-reduced state or the $K_3Fe(CN)_6$-reoxidized state. We cannot exclude the possibility that an Fe–S cluster is present in the enzyme in vivo, and that such a cluster has been lost during the aerobic purification procedure. In this respect, the fact that 50% of the enzyme activity is rapidly lost immediately after disruption of the cells might be of significance.

These are the first EPR spectra of a purified Ni enzyme; the isotope substitution experiment proves that the signal is due to Ni. The spin concentration corresponding to the signal is similar to the enzyme concentration. This agrees well with the Ni contents as determined chemically [2]. Since reduction of the enzyme with H_2 removes the signal without creating others, we propose that the oxidized enzyme contains Ni(III). The g-values and the temperature dependence suggest a $S = 1/2$ state. This is probably created by a tetragonally distorted octahedral ligand field [5,7]. In Ni complexes, the standard redox potential of Ni(III)/(II) is +0.7 to +1.4 V [6,7] and Ni(II) is always the most stable redox state under normal aerobic conditions. This apparently does not hold for Ni in hydrogenase, where Ni(III) is the stable redox state in air.

The finding of redox-active Ni in hydrogenase from *Methanobacterium thermoautotrophicum*, without any EPR signal of an Fe–S cluster strongly suggests the Ni as the site of interaction of H_2 with the enzyme. Further studies are required to obtain additional arguments for this suggestion.

Acknowledgements

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