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Properties of the tungsten-substituted molybdenum formylmethanofuran dehydrogenase from *Methanobacterium wolfei*

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In *Methanobacterium wolfei* two formylmethanofuran dehydrogenases are present, one of which is a molybdenum- and the other a tungsten enzyme. We report here that also the 'molybdenum' enzyme contained tungsten when the archaeon was grown on molybdenum-deprived medium supplemented with tungstate (1 μM). Unexpectedly the tungsten-substituted molybdenum enzyme was catalytically active and displayed a rhombic EPR signal which was attributed to tungsten by the characteristic ^{183}W splitting.

Formylmethanofuran dehydrogenase; Tungsten-substituted molybdenum protein; Tungsten EPR signal; Methanogenic archaea

1. INTRODUCTION

Most methanogenic archaea are dependent on molybdenum for growth [1]. They require this transition metal for the synthesis of formylmethanofuran dehydrogenase which contains molybdenum bound to molybdopterin guanine dinucleotide [2–4]. This enzyme is involved in methane formation from CO_2 and H_2, from formate, methanol and methylamines [5]. Methanogens, which grow on formate, additionally contain a molybdenum formate dehydrogenase [6].

Recently it was found that *Methanobacterium wolfei* can grow equally well on medium supplemented with tungstate instead of molybdate [7]. During growth in the presence of tungstate (1 μM) a second formylethanofuran dehydrogenase was expressed, which contained tungsten bound to molybdopterin guanine dinucleotide. This tungsten formylmethanofuran dehydrogenase differed significantly from the molybdenum formylmethanofuran dehydrogenase in chromatographic behaviour and catalytic properties [4,7].

We report here that during growth of *M. wolfei* in the presence of tungstate this transition metal is also incorporated into the molybdenum formylmethanofuran dehydrogenase. The catalytic and EPR spectroscopic properties of the tungsten-substituted molybdenum enzyme were determined.

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2. MATERIALS AND METHODS

Methanobacterium wolfei (DSM 2970) was grown on medium lacking molybdate and supplemented with tungstate (1 μM) [7]. The formylmethanofuran dehydrogenase was purified from these cells by fast protein liquid chromatography (FPLC) as described previously [7]. The FPLC columns were from Pharmacia (Freiburg, Germany). Formylmethanofuran dehydrogenase activity was assayed with methylviologen as electron acceptor [8] at 65°C in 50 mM Tris-HCl, pH 7.3. Formylmethanofuran was synthesized from methanofuran and 4-nitrophenyl formate [9]. Molybdenum and tungsten were determined via inductively coupled plasma mass spectrometry [7].

EPR spectra were scanned on a Varian E-9 EPR spectrometer equipped with a home-built cryostat. Off- line processing of the spectra was carried out on a personal computer with home-made software. Quantification and simulation of EPR spectra were according to Albracht et al. [10].

3. RESULTS

Formylmethanofuran dehydrogenase was purified from 14 g wet cells of *M. wolfei* grown on molybdate-free medium supplemented with tungstate. The 30 ml cell extract contained 900 U enzyme activity and 1,300 mg protein. Two activity peaks were obtained via FPLC on Mono Q HR. Peak I eluted at the same salt concentration as, described for the molybdenum formylmethanofuran dehydrogenase, and peak II, as described for the tungsten formylmethanofuran dehydrogenase [7].

The activity peak I was further purified by FPLC on phenyl-Superose HR yielding a homogeneous preparation of 150 U with a specific activity of 27 U/mg protein (V_{max}). The purified protein showed the same chromatographic behaviour on Mono Q and phenyl-Superose, the same electrophoretic mobility in native poly-

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acrylamide gels, the same subunit composition after SDS-PAGE (64, 51 and 31 kDa), and the same *N*-terminal amino acid sequence (determined for the 64 kDa subunit) as the purified molybdenum formylmethanofuran dehydrogenase. It contained, however, tungsten (0.3 mol per 146 kDa) rather than molybdenum (<0.01 mol per 146 kDa) and is therefore considered to be the tungsten-substituted molybdenum enzyme (Table 1).

3.1. *Catalytic properties of the tungsten substituted enzyme*

The tungsten-substituted enzyme with 0.3 mol tungsten per mol displayed very similar catalytic properties as the non-substituted molybdenum enzyme with 0.23 mol molybdenum per mol. The apparent V_{max} and the apparent K_m for formylmethanofuran and for methylviologen were almost identical. The two enzymes differed, however, in the apparent V_{max} and apparent K_M for the pseudosubstrate, *N*-furfurylformamide, and in the pH optimum. The temperature optimum of both enzymes was between 65 and 70°C (Table 1).

3.2. *EPR properties of the tungsten-substituted enzyme*

The tungsten-substituted enzyme, as isolated, exhibited at 55 K an EPR signal (not shown) composed of an isotropic signal with g = 2.003 and a second signal with lines at g = 1.925 and 1.875, probably derived from a [4Fe-4S]$^+$ cluster. Upon oxidation of the enzyme solution with air these signals disappeared and a novel rhombic signal with hyperfine splitting appeared (g_{xyz} = 2.0488, 2.0122, 1.9635). The novel signal is shown in Fig. 1, trace A. It represents a spin concentration of 21% of the enzyme concentration and of 63% of the tungsten concentration. In Fig. 1, trace B, a computed EPR signal is depicted, which was calculated (see below) assuming that the rhombic signal is derived from tungsten. Trace C shows a difference spectrum. Fig. 1 strongly suggests that the signal is indeed derived from the transition metal.

The computed signal in Fig. 1, trace B, or Fig. 2, trace C, is a summation of a calculated rhombic S = 1/2 signal (g_{xyz} = 2.0488, 2.0122, 1.9635) without a nuclear hyperfine interaction (Fig. 2, trace A) and the same rhombic signal interacting with a nuclear spin of I = 1/2 (Fig. 2, trace B). Signals A and B were plotted such that their double integrated intensities are related in a ratio of 85.6 to 14.4. This ratio is derived from the natural abundance of the tungsten isotopes: I = 0: ^{180}W, 0.14%; ^{182}W, 26.4%; ^{184}W, 30.6%; and ^{186}W,28,4% and I = 1/2 : ^{183}W, 14.4% [11].

Table 1

Properties of the tungsten-substituted molybdenum formylmethanofuran dehydrogenase in comparison with the non-substituted molybdenum enzyme

Properties	Tungsten-substituted molybdenum enzyme	Non-substituted molybdenum enzyme [4]
Chromatographic behaviour on mono Q and phenyl-Superose	identical chromatographic behaviour	
Apparent molecular mass of the native enzyme	130 kDa	130 kDa
Molecular masses of subunits	64 kDa* 51 kDa 31 kDa	64 kDa* 51 kDa 31 kDa
Transition metal content	0.3 mol W/mol	0.23 mol Mo/mol
Apparent V_{max} with formylmethanofuran	27 U/mg	37 U/mg
Apparent K_m for formylmethanofuran	13 μM	13 μM
Apparent K_m for methylviologen	30 μM	37 μM
Apparent V_{max} with *N*-furfurylformamide	0.1 U/mg	0.35 U/mg
Apparent K_m for *N*-furfurylformamide	1.25 M	53 mM
Temperature optimum	70 °C	70 °C
pH optimum	7.4	7.9

* The *N*-terminal amino acid sequence was found to be MEYIIKNGFVYPLNGVDG(E)

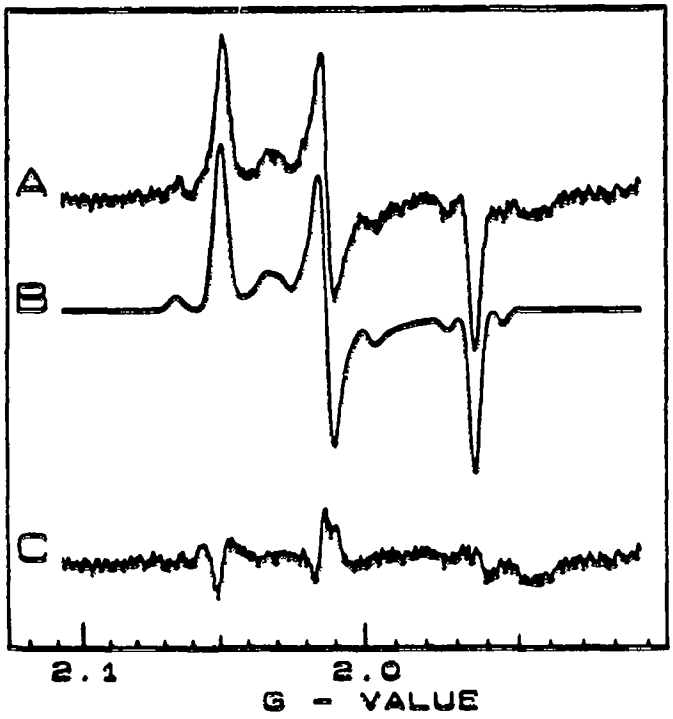

Fig. 1. EPR spectrum of the tungsten-substituted molybdenum formylmethanofuran dehydrogenase from *M. wolfei*. (A) Experimental spectrum after stirring the enzyme solution with air. The sample analyzed contained 41 μM of purified enzyme in 50 mM Tris-HCl, pH 8.75. EPR conditions were: microwave frequence, 9,259 MHz; temperature, 55 K; microwave power incident to the cavity, 2.0 mW; modulation amplitude, 0.32 mT. (B) Simulation of experimental EPR spectrum (trace A) (see Fig. 2). (C) Difference spectrum, A minus B.

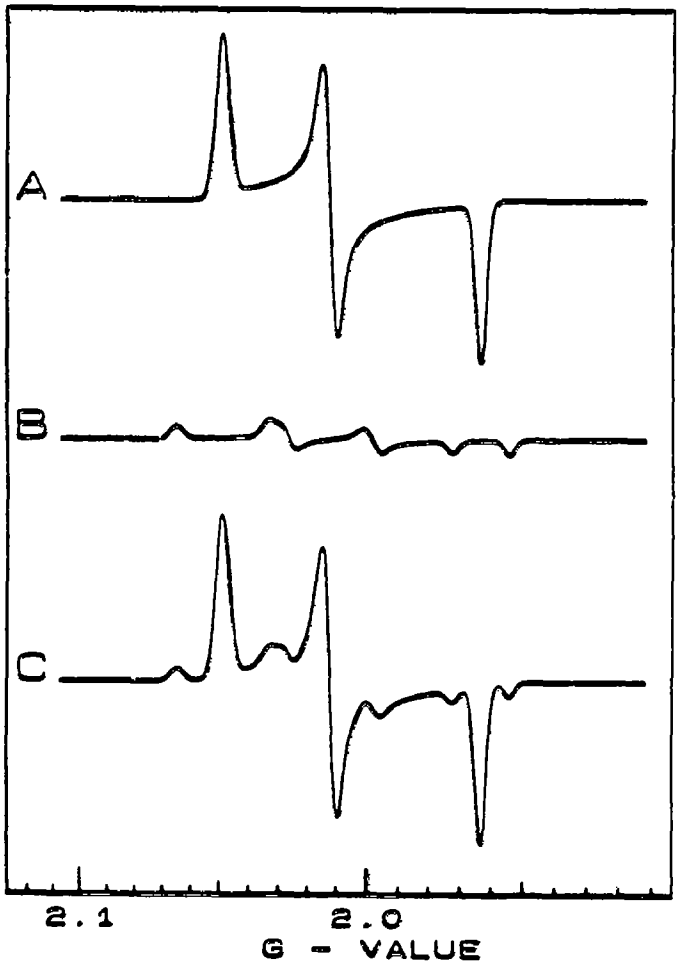

Fig. 2. Computer simulation of the EPR signal exhibited by the tungsten-substituted molybdenum formylmethanofuran dehydrogenase. (A) Computer simulation of the experimental rhombic spectrum (Fig. 1, trace A) as a S = 1/2 signal with parameters g_{xyz} = 2.0488, 2.0122 and 1.9635 and widths(xyz) 0.747, 0.7 and 0.62 mT. (B) Simulation of the same rhombic spectrum but now with hyperfine splitting (nuclear spin I = 1/2): A_{xyz} = 5.G, 4.6 and 3.1 mT. Spectras A and B were plotted such that their double integrated intensities related as 85.6 to 14.4 (see text). (C) Sum of A plus B.

4. DISCUSSION

Previous publications indicate that the biosynthetic replacement of molybdenum by tungsten in molybdenum enzymes generally leads to inactive proteins. This has been shown, for example, for nitrogenase from *Azotobacter vinelandii* [12], nitrate reductase from plants [13], hepatic sulphite oxidase [14], and formate dehydrogenase from *Methanobacterium formicicum* [15]. Rest activity of the enzymes can be attributed to small amounts of molybdenum still present. The tungsten-substituted molybdenum formylmethanofuran dehydrogenase from *M. wolfei* described here was, however, still catalytically active even when more than 95% of the molybdenum was replaced by tungsten. This distinguishes it from all other molybdenum enzymes known to date.

Tungsten and molybdenum both belong to sub-group VI of the periodic table and have almost identical iron radii [16]. This explains in principle why tungsten can substitute for molybdenum in a molybdenum enzyme. The observation that most tungsten-substituted molybdenum enzymes are inactive shows, however, that the ligand field in the active site is generally not constructed so as to allow a functional replacement of molybdenum by tungsten.

The finding that a tungsten-substituted molybdenum enzyme is active was not too surprising in view of the fact that real tungsten enzymes are now known which contain a molybdopterin cofactor and thus have an active site very similar to that of most molybdenum enzymes [17], e.g. formate dehydrogenase from *Clostridium thermoaceticum* [18], *Clostridium formicoaceticum* [19] and *Methanococcus vannielii* [20], carboxylic acid reductase from *C. thermoaceticum* [21] and *C. formicoaceticum* [22], aldehyde:ferredoxin oxidoreductase from *Pyrococcus furiosus* [23] and the tungsten formylmethanofuran dehydrogenase from *M. wolfei* [7].

Indirect evidence is available that molybdenum may partially replace tungsten in the selenium-dependent formate dehydrogenase from *M. vannielii* [20].

EPR spectra have been published from tungsten-substituted nitrogenase from *A. vinelandii* [12], hepatic sulfite oxidase [14], and the tungsten enzyme, formate dehydrogenase, from *C. thermoaceticum* [24]. The EPR signals of these enzymes were much more complex than the signal displayed by the tungsten-substituted formylmethanofuran dehydrogenase which is clearly derived from tungsten, as evidenced by characteristic ^{183}W isotope splitting. This is to the best of our knowledge the first report on an EPR signal in a functional tungsten-containing protein which can unambiguously be assigned to tungsten.

The tungsten EPR signal is probably derived from W(V) since W(IV) and W(VI) are EPR silent. The finding that the tungsten signal appeared only after oxidation of the purified enzyme with air suggests that the tungsten-substituted molybdenum formylmethanofuran dehydrogenase, as isolated, contained the transition metal in the W(IV) oxidation state. In molybdenum enzymes the oxidation state of molybdenum changes between Mo(IV), Mo(V), and Mo(VI) [17]. Such a change in oxidation state is therefore also postulated for tungsten in the active tungsten-substituted molybdenum enzyme.

The tungsten EPR signal displayed two g-values above 2.0 which is rather unexpected. It has been shown, however, that it becomes possible for a tungsten(V) complex with a low energy charge transfer excited state to have g values > 2.0 [24].

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