Formate dehydrogenase from *Clostridium pasteurianum*

Electron paramagnetic resonance spectroscopy of the redox active centers

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Formate dehydrogenase from *Clostridium pasteurianum* catalyses the interconversion of CO_2 and formate. It is a complex enzyme, containing molybdenum, iron, acid-labile sulfur and pterin. Redox potentiometry and EPR analysis reveal two spectrally and thermodynamically distinct iron-sulfur clusters, with g 1.87, 1.95 and 2.05 (E_{m8} −318 mV) and g 1.92 and 2.05 (E_{m8} −372 mV), both present at approx. 0.3 spins per enzyme. There is also a free radical signal centered near g 2.005 which we attribute to the pterin, perhaps in its anionic semiquinone form. This signal disappears on oxidation (E_{m8} −150 mV), but is not reduced further at pH 8. It is present at approx. 0.8 spins per enzyme. No EPR signals attributable to the molybdenum were detected.

Formate dehydrogenase *Iron-sulfur protein* *Pterin* *Moco*

1. INTRODUCTION

Formate dehydrogenases are a diverse group of enzymes found in both prokaryotes and eukaryotes, capable of interconverting CO_2 and formate. The enzymes from different sources differ markedly in their size, metal content and catalytic activity (reviewed in [1]). Despite the widespread occurrence of formate dehydrogenases, the ability to assimilate CO_2 to formate is an uncommon pathway restricted to a few anaerobes, and in these the enzyme is sometimes known as CO_2 reductase [1]. Such an enzyme has been purified from *Clostridium thermoaceticum* [2]; it is a large $\alpha_2\beta_2$ complex, M_r 340000, contains 2 W, 36 Fe, 50 S and 2 Se atoms per molecule, and uses NADPH as an electron donor. In contrast, the enzyme from *C. pasteurianum* [3] has $\alpha_1\beta_1$ structure, M_r 117000, contains 2 Mo, 24 Fe and 28 S atoms per molecule, and receives electrons from ferredoxin. Twenty of the irons can be extruded as Fe_4S_4 clusters [3]. The molybdenum seems to be in the form of the molybdenum-pterin cofactor (known as Moco) [3,4], in that extracts from the *C. pasteurianum* enzyme were able to reconstitute nitrate reductase activity in the *Neurospora crassa* nit-1 mutant which lacks the ability to make the cofactor [5,6]. However, the molybdenum in the *C. pasteurianum* formate dehydrogenase lacks the Mo-S bonds found in other Moco containing enzymes, at least in the unreduced forms studied to date [7], and does not seem to undergo oxidation/reduction during catalysis [3].

In this paper we examine the EPR properties of the enzyme.

2. MATERIALS AND METHODS

Formate dehydrogenase was purified from *C. pasteurianum* W5 as described [3], with all operations carried out under strictly anaerobic conditions in the presence of 2 mM dithiothreitol. The activity of the enzyme was measured spectrophotometrically with formate as the substrate

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and methyl viologen as the electron acceptor [3]. Redox potentiometry was carried out as described by Dutton [8], with 250 μl samples being withdrawn from the titration cell, placed in 3 mm i.d. EPR tubes, and rapidly frozen in chilled isooctane. EPR measurements used a Varian E109 spectrometer equipped with an Oxford Instruments liquid helium cryostat. Spin quantitation used a Cu^{2+} standard.

3. RESULTS

The enzyme as prepared in the presence of dithiothreitol exhibits a pronounced free-radical EPR signal near $g = 2$ (fig.1). Under non-saturating conditions (fig.1A) this signal exhibited a zero-crossing near $g = 2.003$, with $\Delta_H = 1.44$ mT, and pronounced 'wings' on the shoulders of the signal. This signal could still be seen at temperatures above 250 K. When the temperature was lowered to 6 K, and the microwave power increased to partially saturate the signal, the apparent g value increased to $g = 2.005$, and the signal lost its wings and narrowed to 1.25 mT (fig.1B). Under non-saturating conditions this signal integrated to 0.8 spins per molecule. Redox titrations indicated an E_{m8} of -150 mV, with the signal visible only in the reduced form (fig.2). The unusual temperature and power dependence revealed in fig.1 were seen throughout the titration. This free-radical signal was unaffected by the addition of the substrate formate.

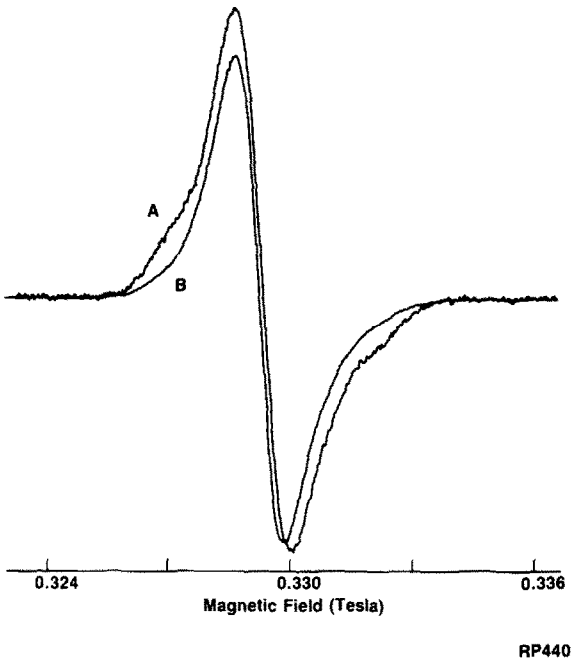

Fig.1. EPR signals near $g = 2$ in formate dehydrogenase. Enzyme (7.2 mg/ml) was dissolved in 100 mM Tris buffer, pH 8, in the presence of 2.5 mM dithiothreitol which poised the E_h near -350 mV. For A the temperature was 23 K with 0.5 mW of applied microwave power; B was recorded at 6.6 K with 10 mW of applied power. The modulation amplitude was 0.08 mT.

The enzyme also shows EPR spectra reminiscent of iron-sulfur clusters, as shown in fig.3. In contrast to the free-radical signal, these are only seen below about 30 K, suggesting that they originate in Fe_4S_4 clusters. Redox titrations reveal two thermodynamically and spectroscopically distinct species, both present at about 0.3 spins per enzyme. One has apparent g values of 1.87, 1.95 and 2.05, and E_{m8} of -318 mV; the other is more axial with apparent g values of 2.05 and 1.92, and E_{m8} of -372 mV (fig.4). As can be seen in fig.3, the former is reduced in the enzyme as prepared, while the latter is reduced by the substrate formate.

The activity of the enzyme was measured as each sample was taken during the redox titrations of

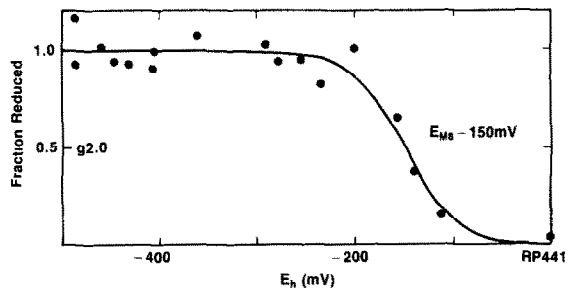

Fig.2. Redox titration of the signal near $g = 2$ in formate dehydrogenase. Enzyme (3 mg/ml) was dissolved in 65 mM Tris buffer, pH 8, in the presence of 40 μM safranin, *o*-naphthoquinone, *p*-benzoquinone, duroquinone, indigo di- and trisulfonate, *o*-naphthoquinone-4-sulfonate, 9,10-anthraquinone-2-sulfonate and 9,10-anthraquinone-1,5-disulfonate. These redox mediators have been demonstrated not to interfere with EPR measurements near $g = 2$ [13]. Dithiothreitol was added at the beginning of the experiment to 125 μM, and the titration performed with either sodium dithionate or potassium ferricyanide. EPR conditions as in fig.1A.

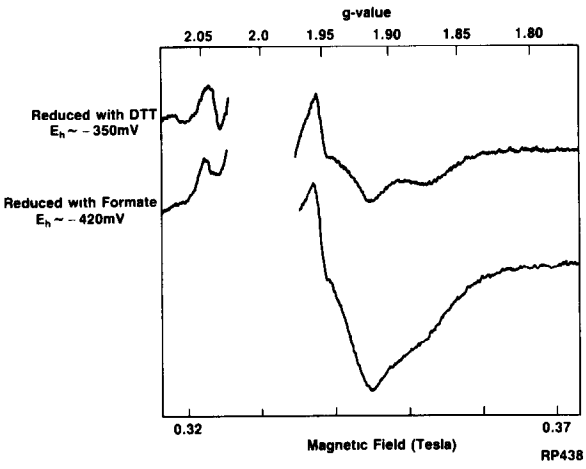

Fig.3. EPR signals attributable to iron-sulfur clusters in formate dehydrogenase. Samples were prepared as in fig.1 with either dithiothreitol (DTT) or formate (15 mM) as indicated. Spectra were obtained at 22.5 K with 10 mW of applied microwave power and a modulation amplitude of 0.8 mT.

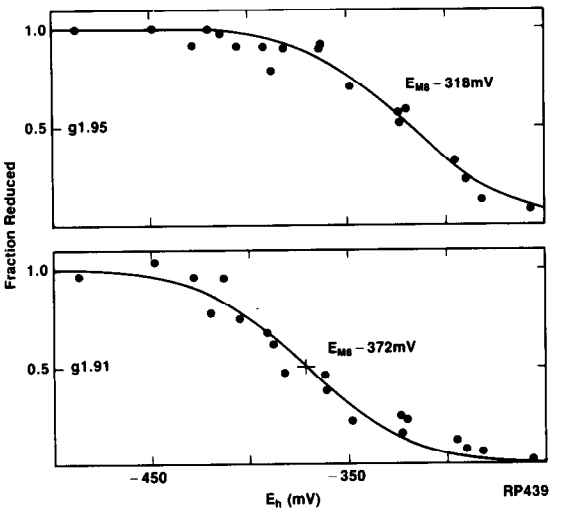

Fig.4. Redox titrations of the iron-sulfur clusters of formate dehydrogenase. Samples were prepared as for fig.2 with the addition of 40 μM of methyl and benzyl viologens. EPR measurements were made as in fig.3.

figs 2 and 4. These titrations were started with the enzyme in the presence of 125 μM dithiothreitol (and the redox mediators listed in the figure legends) which poised the system near E_h −350 mV (pH 8). Under these conditions, samples removed for assay of enzyme activity showed fully active enzyme (see [3]). While excess dithionite irreversibly destroys enzyme activity [3], controlled additions to lower the potential as shown in figs 2 and 4 had no inhibitory effect. In oxidative titrations the enzyme activity was stable until the higher potential iron sulfur cluster started to titrate, whereupon activity began to decline. The loss of activity was not a Nernstian phenomenon, but rather seemed to be a function of both potential and time. For example, 75% of the activity was lost after 1 h at E_h (pH 8) −200 mV. The loss of activity was irreversible, but the disappearance of the EPR signals was not; these titrated in a fully reversible manner regardless of whether the enzyme was fully active, or totally inactive.

4. DISCUSSION

The EPR properties of formate dehydrogenase from *C. pasteurianum* discussed in this paper are clearly rather different both from other formate dehydrogenases and from other enzymes containing Moco (see [1]). The enzyme exhibits no EPR spectrum that can be attributed to molybdenum, which is in contrast to all other known Moco containing enzymes, but does exhibit a free-radical signal that is reminiscent of some flavin species [9,10]. Flavin has not been identified in the enzyme, but pterin has been identified by its fluorescent properties after extraction [3]. Pterins are closely related chemically to flavins, so it seems reasonable to propose that the free-radical of fig.1 arises from a pterin. By analogy with measurements on flavins [9,10], the relatively narrow linewidth of the signal suggests that the radical is an anionic semiquinone, but this needs to be examined further, perhaps by examining the pH-dependence of the E_m.

Chemical analysis reveals 24 non-heme irons per enzyme, of which >20 can be extruded as Fe_4S_4 clusters [3]. Yet EPR reveals only two EPR signals that might arise from the reduced form of such centers. This is not unusual in such complex enzymes; for example the molybdenum-iron protein of nitrogenase contains about 32 iron atoms per enzyme, of which at least 16 are in the form of Fe_4S_4 clusters [11]. Mossbauer spectroscopy suggests that these are ferredoxin-like centers, but the enzyme exhibits no EPR signals attributable to such centers in the dithionite reduced enzyme [11].

Similarly the rather low spin-quantitation of the two iron-sulfur clusters reported here is not unusual in such complicated enzymes. Of course it is possible that the 0.3 spins per total enzyme reflects the concentration of fully active enzyme in a catalytically heterogeneous preparation (which is electrophoretically homogeneous, see [3]), but it is also possible that some of the clusters are in an alternative spin-state that has not been detected here. For example, even the most active preparations of the nitrogenase iron protein exhibit an EPR spectrum equivalent to only about 0.3 spins per molecule (see [12]).

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