Purification and properties of formate dehydrogenase from *Pseudomonas aeruginosa*

Electron-paramagnetic-resonance studies on the molybdenum centre

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Formate dehydrogenase from *Pseudomonas aeruginosa* contains molybdenum, a [4Fe–4S] cluster and cytochrome *b*. This paper reports the detection of molybdenum as Mo(V) by e.p.r. spectroscopy. In order to generate Mo(V) signals, addition of amounts of excess formate varying between 10- and 50-fold over enzyme, followed by 200-fold excess of sodium dithionite, were used. Two Mo(V) species were observed. One, the major component, has $g_1 = 2.012$, $g_2 = 1.985$ and $g_3 = 1.968$, appeared at low concentrations of formate and increased linearly in intensity with increasing concentrations of formate up to 25-fold excess over the enzyme. At higher formate concentration this signal disappeared. The appearance and disappearance of this Mo(V) signal seems to parallel the state of reduction of the [4Fe–4S] clusters. A second, minor, Mo(V) species with g -values $g_1 = 1.996$, $g_2 = 1.981$ and $g_3 = 1.941$ appears at a constant level during the formate–dithionite titration. No evidence has been obtained for nuclear hyperfine coupling to protons. The major Mo(V) species has unusual e.p.r. signals compared with other molybdenum-containing enzymes, except for that observed in the formate dehydrogenase from *Methanobacterium formicicum* [Barber, Siegel, Schauer, May & Ferry (1983) *J. Biol. Chem.* **258**, 10839–10845]. The present work suggests that the enzyme is acting as a CO₂ reductase, with dithionite as an electron donor to a [4Fe–4S] cluster, which in turn donates electrons to molybdenum, producing a Mo(V) species with CO₂ bound to the metal.

INTRODUCTION

A variety of formate dehydrogenases (FDH) have been isolated to varying degrees of purity from several sources, including aerobic (Müller *et al.*, 1978) and anaerobic (Yamamoto *et al.*, 1983) bacteria. The non-protein components of the enzyme may include any one, or a combination, of molybdenum, selenium, tungsten, iron, labile sulphur and flavin; the requirement for these metals in the enzyme is most often established by nutritional studies (Wagner & Andreessen, 1977). A small number of FDHs have also been studied by direct methods. Thus the FDH from *Escherichia coli* was found to contain cytochrome *b*, selenium, iron–sulphur clusters and molybdenum (Enoch & Lester, 1975). However, few FDHs have been purified to homogeneity, and therefore spectroscopic studies of the metal centres have been limited.

The predominant physiological role of FDHs is to oxidize formate to CO₂, as in the case in *E. coli*. However, the FDH isolated from a number of anaerobes, such as the *Clostridium* species, have the ability to reduce CO₂ to formate. These enzymes are then known as ‘CO₂ reductases’ (Jungermann *et al.*, 1970). The formate produced may subsequently be metabolized into one carbon-atom precursors of cell materials such as methionine and acetic acid (Ljungdhal & Wood, 1969).

Although many FDHs are reported to contain molybdenum, and to require molybdate in the growth medium for the formation of active enzyme, surprisingly

few have so far exhibited e.p.r. signals attributable to a molybdenum centre. This is in sharp contrast with the situation in other molybdenum-containing enzymes, where the Mo(V) signals are readily generated or often observed in the enzyme as isolated (Bray, 1980). The most complete molybdenum e.p.r. studies yet reported of FDH are from those on the methanogenic bacterium *Methanobacterium formicicum* (Barber *et al.*, 1983). The e.p.r. signals due to Mo(V) species in the spectrum of the dithionite-reduced FDH exhibits a g_{av} greater than 2.0, the largest yet reported for molybdenum in a protein.

More recently (Prince *et al.*, 1985), e.p.r. studies of the FDH from *Clostridium pasteurianum* failed to exhibit signals from the molybdenum centre. Those authors suggested this is due to the failure of molybdenum centre to undergo redox changes during catalysis, thus Mo(V) was not being generated.

Following the low-temperature e.p.r. and magnetic-c.d. studies of the haem and iron–sulphur centres in the FDH from *Pseudomonas aeruginosa* in the preceding paper (Godfrey *et al.*, 1987), we report here the e.p.r. spectra of this enzyme in response to reduction by formate and sodium dithionite in an attempt to identify the molybdenum centre of the enzyme.

EXPERIMENTAL PROCEDURES

FDH was purified from *Pseudomonas aeruginosa* as described in the preceding paper (Godfrey *et al.*, 1987). Enzyme samples for e.p.r. analysis used in this study were

all 150 μM in haem and were buffered in 100 mM-Mes, pH 6.0, Hepes, pH 7.8, or Ches, pH 9.0. Sodium formate and sodium dithionite were freshly prepared in rigorously degassed buffer. All additions were performed anaerobically in a glove box (Faircrest Engineering, Croydon, Surrey, U.K.) under an atmosphere of < 2 p.p.m. O_2 . The enzyme sample used in this study showed a high FDH activity (760 units) (Godfrey *et al.*, 1987) and also a small amount of NiR activity (487 nmol/min per mg compared with 10–50 $\mu\text{mol}/\text{min}$ per mg for fully active NiR).

E.p.r. spectra were recorded on a Bruker ER-200D X-band spectrometer operating at 100 kHz modulation and 0.63 mT (6.3 G) field modulation. Spectra recorded at 10 K and 100 K were obtained by using an Oxford Instruments (ESR-9) helium-flow cryostat.

RESULTS AND DISCUSSION

Presented in Fig. 1 are the e.p.r. spectra of *P. aeruginosa* FDH reduced with various concentrations of sodium formate. Up to formate concentrations of ~ 2 mM, very little change is apparent in the e.p.r. spectrum. The spectrum of the enzyme in the oxidized state, and in the presence of 2 mM-formate, exhibits a

Fig. 1. E.p.r. spectra of FDH (*P. aeruginosa*) showing the effect of reduction by sodium formate

The enzyme concentration was 150 μM in 100 mM-Hepes/NaOH buffer, pH 7.8. Formate concentrations were: (i) 2 mM, (ii) 8 mM, and (iii) 10 mM. Spectrometer settings: frequency 9.41 GHz; microwave power 2.01 mW; gain 5×10^5 ; sample temperature 10 K.

near-axial signal at $g = 2.01$, probably arising from an oxidized [3Fe-4S] cluster. This signal is present to various extents in our preparations of FDH and has been assigned to a [3Fe-4S] cluster arising from a contamination by NiR (see Godfrey *et al.*, 1987). Also present in this spectrum is an unassigned signal at $g = 2.05$ which may result from adventitious copper. At higher levels of formate reduction (8 mM), the $g = 2.01$ signal is much reduced, and e.p.r. signals typical of [4Fe-4S] clusters become apparent. These signals described previously in preparations of FDH (Godfrey *et al.*, 1987) have been assigned to two [4Fe-4S] clusters. The first arises from FDH itself, with g -values of $g_1 = 2.044$, $g_2 = 1.943$ and $g_3 = 1.903$. The second reduced [4Fe-4S] species apparent in this spectrum is due to the minor contamination with NiR seen in other preparations to various extents. The g -values of this species are $g_1 = 2.046$, $g_2 = 1.945$ and $g_3 = 1.87$. The near similarity of the g_1 and g_2 values of these two species makes the separation of these signals impossible in this region. However, the g_3 -values are sufficiently different to allow their distinction. Addition of larger concentrations of formate, 10 mM and above, has no further effect on the e.p.r. features assigned to the reduced [4Fe-4S] clusters. However, a new signal at $g = 2.0$ appears which probably arises from a free-radical species (Godfrey *et al.*, 1987).

During a number of titrations of FDH with both sodium formate and sodium dithionite we have been unable to produce a state of the enzyme exhibiting e.p.r. signals ascribable to Mo(V). However, during an experiment to determine the extent of reduction of an FDH sample with formate, to which an excess of dithionite was subsequently added, Mo(V) e.p.r. signals were observed. The results of a second experiment to observe this phenomenon are shown in Fig. 2. In an attempt to generate the Mo(V) signals, various amounts of formate were added to FDH before the addition of a large excess (20 mM) of dithionite. The e.p.r. spectra observed at low temperature (10 K) are shown in Fig. 2(a). In the presence of low levels of formate (≤ 2 mM) the e.p.r. spectrum of FDH shows little change from the oxidized spectrum, (cf. Fig. 1). The addition of 20 mM-dithionite at these levels of formate produces complete reduction of the [4Fe-4S] clusters. At higher levels of formate the extent of reduction of the [4Fe-4S] clusters, as judged by the e.p.r. spectrum, is, rather unexpectedly, less than that described above. Thus, in the presence of 4 mM-formate and 20 mM-dithionite, there is only a small ($\sim 20\%$) extent of reduction of the [4Fe-4S] clusters. With the addition of higher concentrations of formate (≥ 8 mM), followed by 20 mM-dithionite, the [4Fe-4S] clusters are once more reduced. The high-temperature (100 K) e.p.r. spectra are shown in Fig. 2(b). These show the appearance of rhombic signals around $g = 2$, not previously seen in the presence of formate or dithionite alone. These we assign to Mo(V). The major species present exhibits g -values of $g_1 = 2.012$, $g_2 = 1.985$ and $g_3 = 1.968$. This signal appears at low concentrations of formate (2 mM) and increases linearly in intensity with increasing concentration of formate up to 4 mM. However, at higher formate concentrations (8 mM), this signal completely disappears. The appearance and disappearance of this Mo(V) signal at 100 K seems to parallel directly the state of reduction of the [4Fe-4S] clusters as judged by the e.p.r. recorded at 10 K.

Also present in the spectra shown in Fig. 2(b) is a

Fig. 2. E.p.r. spectra of FDH (*P. aeruginosa*) showing the effect of addition of formate followed by an excess of sodium dithionite (20 mM)

The enzyme concentration and buffer were as in Fig. 1. Formate concentrations were: (i) 2 mM, (ii) 3 mM, (iii) 4 mM, and (iv) 8 mM. Spectrometer settings were as for Fig. 1. The sample temperatures were 10 K (a) and 100 K (b).

second rhombic species also postulated to arise from Mo(V), exhibiting $g_1 = 1.996$, $g_2 = 1.981$ and $g_3 = 1.941$. This minor species, although not previously observed, appears at a constant level during this formate–dithionite titration.

Studies of the Mo(V) signals of nitrate reductase from *Escherichia coli* (Vincent & Bray, 1978) and also from *P. aeruginosa* (Godfrey *et al.*, 1984) have shown the presence of two types of Mo(V) e.p.r. spectrum. Since transitions between the two forms can be accomplished by changes of pH, they are referred to as the ‘low-pH’ and the ‘high-pH’ signal. It has more recently been shown that the pK for interconversion is raised in the presence of various anions (George *et al.*, 1985). The low-pH form apparently exists as a series of complexes with different anion ligands of molybdenum, including Cl^- , F^- , NO_3^- and NO_2^- . The high-pH form of the enzyme does bear a coupled proton, but with a much weaker coupling. It has been postulated that this proton is not identical with the proton whose dissociation controls interconversion between the two species, but may instead be located in the protein rather than attached to a ligand of molybdenum.

The Mo(V) signals from FDH presented in Fig. 2(b) and recorded at pH 7.8 fail to show any evidence of hyperfine coupling to a proton. This result also contrasts with the report that the Mo(V) signals of FDH from

Methanobacterium formicicum exhibits nuclear hyperfine interaction with two equivalent protons (Barber *et al.*, 1983). In an attempt to observe proton hyperfine structure of the Mo(V) signal we have studied the effect of pH on the e.p.r. spectra of FDH under the conditions required to generate Mo(V) at pH 7.8 (3 mM-formate + 20 mM-dithionite). The 10 K e.p.r. spectra of FDH under these conditions at pH 6.0, 7.8 and 9.0 are illustrated in Fig. 3(a). It is clear that the extent of reduction of the [4Fe–4S] clusters is dependent on pH, being greater at pH 6.0 and 9.0 than at pH 7.8. This pH-dependence is also apparent in the high-temperature (100 K) spectra (Fig. 3b). At pH 9.0 the Mo(V) signals are difficult to observe, whereas at pH 6.0 the signal is observable, although much smaller in intensity than at pH 7.8. However, even at pH 6.0 the Mo(V) signals fail to display any proton hyperfine structure. We have not investigated the effect of halide-ion binding to see whether this will elicit a proton-coupled signal.

In order to determine whether the addition of formate to the above samples would bring about further changes to the e.p.r. spectrum, a second aliquot of formate was added to the pH 6.0 and 9.0 samples. At pH 6.0 the signals arising from the reduced [4Fe–4S] cluster (Fig. 4a) are affected only slightly, whereas at pH 9.0 the addition of a second aliquot of formate leads to an increase in the amount of reduced [4Fe–4S] present. The changes in the

Fig. 3. E.p.r. spectra of FDH (*P. aeruginosa*) in the presence of 3 mM-formate at 20 mM-dithionite at various pH values

The enzyme concentration was 150 μ M in the following buffers: (i) 100 mM-Mes, pH 6.0, (ii) 100 mM-Hepes, pH 7.8, and (iii) 100 mM-Ches, pH 9.0. Spectrometer settings are in Fig. 1. Sample temperatures were 10 K (a) and 100 K (b).

high-temperature (100 K) spectra are more dramatic; thus at pH 6.0 the addition of extra formate gives rise to a spectrum composed purely of the major species described in Fig. 2(b). This spectrum is extremely well resolved and shows clearly the lack of any observable proton hyperfine interaction. At pH 9.0 the addition of more formate has quite a different effect; a mixture of the two Mo(V) species are produced. However, the minor species of Fig. 2(b) is now predominant. It seems unlikely that this reflects any pH-dependence of the Mo(V) signal.

Conclusions

Reductive titrations of FDH from *P. aeruginosa* using both sodium formate and sodium dithionite have failed to produce e.p.r. signals attributable to the molybdenum centre of this enzyme. However, we have shown in the present paper conditions under which the Mo(V) signals can be generated. Indeed, this appears to be only the second demonstration of the molybdenum centre in any FDH using e.p.r. spectroscopy. The precise conditions necessary to generate Mo(V) are uncertain, but in our hands the maximum signal intensity was observed when the enzyme was titrated with an amount of formate sufficient to reduce the contaminating [3Fe-4S] cluster, but insufficient to reduce the [4Fe-4S] cluster of FDH (≤ 4 mM), followed by addition of an excess of dithionite (20 mM). Under these conditions, at pH 7.8, the Mo(V) signals are readily generated. These results conclusively

demonstrate that molybdenum is present in FDH from *P. aeruginosa* and that Mo(V) is an accessible oxidation state in this enzyme.

The Mo(V) e.p.r. signals generated under the conditions described are unusual in appearance compared with those of other molybdenum-containing enzymes. Firstly, the g_1 value of 2.012 is larger than that commonly observed (Bray, 1980). However, this is lower than observed for the FDH from *Methanobacterium formicicum* (Barber *et al.*, 1983). Also the extent of rhombicity (Δ) calculated from:

$$\Delta = (g_2 - g_3)/(g_1 - g_3) = 0.386$$

is very close to the value of Δ (0.375) obtained for the *Methanobacterium* enzyme. Secondly, the Mo(V) signal does not exhibit any proton hyperfine coupling nor could we produce a low-pH species. Recent reports of the NiR from *E. coli*, a molybdoenzyme, have suggested a complex relationship between the high-pH and low-pH species and the effect of the Cl^- content of the medium (George *et al.*, 1985). Thus it is possible to produce samples of the enzyme as prepared in Hepes buffer (pH 7, without added Cl^-) that give only the high-pH signal. Examination of the same enzyme in the presence of formate showed that it exhibited proton coupling to this anion. The absence of this coupling in *P. aeruginosa* FDH suggests either that the formate ion is not bound to the molybdenum, or alternatively that CO_2 is bound.

Fig. 4. E.p.r. spectra of FDH (*P. aeruginosa*) showing the effect of further addition of formate (4 mM) on the spectra shown in Fig. 3(a)

(i) pH 6.0; (ii) pH 9.0. Sample temperatures were 10 K (a) and 100 K (b). See the legend to Fig. 3 for further details.

In support of the latter hypothesis is the special condition under which the Mo(V) signals are generated. The intermediate levels of formate required do not reduce the [4Fe-4S] clusters or molybdenum of FDH, but when dithionite is subsequently added, Mo(V) is generated, although the [4Fe-4S] clusters are not reduced. This suggests that the enzyme is acting as a CO₂ reductase, with dithionite acting as an electron donor to the [4Fe-4S] clusters, which in turn donates electrons to molybdenum, producing an Mo(V) species with CO₂ bound at the metal. This mechanism is consistent with the absence of proton hyperfine coupling to the substrate.

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