Purification and properties of formate dehydrogenase from *Pseudomonas aeruginosa*

Characterization of haem and iron–sulphur centres by magnetic-circular-dichroism and electron-paramagnetic-resonance spectroscopy

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The purification of formate dehydrogenase (FDH) from *Pseudomonas aeruginosa* after anaerobic growth on nitrate-containing medium was carried out. The separation of the FDH enzyme from nitrate reductase (NiR), which are found together in a particle fraction and constitute the short respiratory chain of this bacterium, has been followed by optical, magnetic c.d. (m.c.d.) and e.p.r. spectroscopy. These techniques have allowed the haem, iron–sulphur clusters and molybdenum components to be detected and, in part, their nature to be determined. Attempts to extract FDH anaerobically in the absence of sodium dithionite led to loss of activity. Addition of sodium dithionite maintained the activity of the enzyme, even after subsequent exposure to air, in an assay involving formate reduction with Nitro Blue Tetrazolium as reductant. Three preparations of FDH have been examined spectroscopically. The preparations vary in the amount of contaminating nitrate reductase, the amount of cytochrome *c* present and the concentration of oxidized [3Fe–4S] cluster. Optical spectra and low-temperature m.c.d. spectroscopy show the loss of a cytochrome-containing protohaem IX co-ordinated by methionine and histidine as NiR is separated from the preparation. In its purest state FDH contains one molecule of cytochrome co-ordinated by two histidine ligands in the oxidized state. This cytochrome has an e.p.r. spectrum with $g_z = 3.77$, the band having the unusual ramp shape characteristic of highly anisotropic low-spin ferric haem. It also shows a charge-transfer band of high intensity in the m.c.d. spectrum at 1545 nm. It has recently been shown [Gadsby & Thomson (1986) FEBS Lett. 197, 253–257] that these spectroscopic properties are diagnostic of a bishistidine co-ordinated haem with steric constraint of the axial ligands. The e.p.r. and m.c.d. spectra of the reduced state of FDH reveal the presence of an iron–sulphur cluster of the [4Fe–4S]⁺ type. The *g*-values are 2.044, 1.943 and 1.903. An iron–sulphur cluster of the class [3Fe–4S], detected by e.p.r. spectroscopy in the oxidized state and by low-temperature m.c.d. spectroscopy in the reduced state, is purified away with the NiR. Finally, an e.p.r. signal at $g = 2.0$ with a narrow bandwidth which persists to 80 K is observed in the purest preparation of FDH. This may arise from an organic radical species.

INTRODUCTION

When *Pseudomonas aeruginosa* is grown anaerobically under conditions of nitrate respiration, formate donates electrons to nitrate via a relatively short electron-transport chain consisting of two complex multi-centred metalloenzymes, namely nitrate reductase (NiR) and formate dehydrogenase (FDH). This chain appears to be very similar to that first characterized by Enoch & Lester (1974, 1975) in *Escherichia coli* when the latter is growing anaerobically under conditions of nitrate respiration. By extracting particles from *E. coli* cells, Itagaki *et al.* (1962) succeeded in obtaining soluble FDH, cytochrome *b*₁ and NiR. The process had to be performed under anaerobic conditions owing to the extreme sensitivity of the FDH to oxygen. Ruiz-Herrera & De Moss (1969) obtained evidence for the specific cytochrome *b*₁ components in the FDH–NiR system. Enoch & Lester (1974, 1975) obtained a purified preparation of FDH containing a

globular-protein–detergent complex free of phospholipid with a polypeptide subunit structure $\alpha_4\beta_4\gamma_2$ having an M_r of $608\,000 \pm 60\,000$. The enzyme was also shown to contain, in relative molar amounts: haem, 1.0; molybdenum, 0.95; selenium, 0.96; non-haem iron, 14; acid-labile sulphide, 13. It was suggested that this molecular unit is one of the tetrameric subunits α or β . No spectroscopic characterization of the metal centres in FDH from *E. coli* appears to have been reported.

We have been engaged in the preparation and characterization of the metal components of the electron-transport chain of the NiR–FDH complex in *P. aeruginosa*. During the course of the preparation of the NiR, a fraction with a high FDH activity was observed. This has led us to purify this enzyme and to carry out a characterization of the three classes of metal centres, namely, molybdenum, haem and iron–sulphur clusters using e.p.r. and m.c.d. spectroscopy. A comparison has been made between the spectroscopic properties of three

Abbreviations used: m.c.d., magnetic c.d.; FDH, formate dehydrogenase; NiR, nitrate reductase; NBT, Nitro Blue Tetrazolium; β , Bohr magneton; *B*, magnetic-field strength [in T(tesla)]; *k*, Boltzmann constant; *T*, temperature.

different samples, one of which contains both NiR and FDH activity. In this way we have been able to monitor the fractionation of the major metal components into the FDH and NiR enzymes. The present paper describes the identification of the haem and iron-sulphur components of FDH, primarily by using a combination of e.p.r. and m.c.d. spectroscopy. The following paper (Gadsby *et al.*, 1987) describes the Mo(V) species present in FDH. This is only the second report of the Mo e.p.r. signals in FDH, the first being the FDH from *Methanobacterium formicum* (Barber *et al.*, 1983).

Finally, in the third of this series of papers (Godfrey *et al.*, 1987), a spectroscopic study of the particle is presented and a sequence is proposed for the components of the electron-transfer chain within the complex.

MATERIALS AND METHODS

Assay procedures

FDH activity was determined with NBT as described by Enoch & Lester (1975). Protein was determined by the biuret method. One unit of FDH activity is defined as the reduction of 1 μmol of NBT/min per mg of protein at 30 °C. Nitrate reductase activity was measured colorimetrically by the method of MacGregor *et al.* (1974). Mo was estimated with a dithiol reagent (Hart *et al.*, 1970). Cytochrome was estimated by the pyridine-haemochrome method (Appleby, 1969), using an ϵ_{557} value of $32 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

Analytical gel filtration

M_r was estimated by gel filtration on a calibrated Sephacryl S-300 column (40 cm $\times$ 2.5 cm). This was equilibrated in 50 mM-potassium phosphate, pH 8.2. Triton X-100 (0.1%) was included for the sample run only. Calibration proteins were cytochrome *c* (M_r 12380), malate dehydrogenase (70000), catalase (232000), apoferritin (443000) and thyroglobulin (669000). The void volume (V_o) was determined with Blue Dextran (2000000), and the total gel volume (V_t) was calculated mathematically. The elution volume (V_e) is proportional to $\log M_r$ (Andrews, 1965). Relative elution positions were calculated by the relation:

$$k_{av.} = (V_e - V_o)/(V_t - V_o)$$

where $k_{av.}$ is proportional to $\log M_r$. Typically a 2.5 ml sample was applied and was eluted at 20 ml/h. FDH was assayed as described above, and marker proteins were monitored at 280 nm.

Electrophoresis

Non-denaturing polyacrylamide-gel electrophoresis was performed on 5% gels as described by Laemmli (1970), except that 0.1% Triton X-100 was added to the buffer system. Two gels were run in tandem, one stained for protein with Coomassie Brilliant Blue and the other tested for FDH activity. This was done by soaking the gel in approx. 50 ml of sodium phosphate buffer (50 mM), pH 7.3, containing 0.1 mM-NBT and 2 mM-sodium formate, for 5–10 min. FDH activity appeared as one or more dark-blue bands.

Culture of *Pseudomonas aeruginosa*

Samples of *Pseudomonas aeruginosa* N.C.T.C. 6750 were obtained from the National Collection of Type Cultures, Colindale, London NW9 5HT, U.K. Cultiva-

tion was performed in a nitrate complex growth medium (Soininen & Ellfolk, 1975) supplemented with 1 μM -sodium molybdate and 1 μM -sodium selenite. The medium (40 litres), contained in a stainless-steel barrel fitted with a New Brunswick head, was inoculated with 300 ml of a starter culture. The contents of the barrel were stirred, and anaerobic conditions were ensured by bubbling a continuous stream of N_2 through the medium. The pH was maintained between 6.9 and 7.0 by means of a pH-stat, as previously described (Foote *et al.*, 1983). Growth is complete after 18–20 h at 37 °C. Thereafter the cells were concentrated to about 2 litres by using a Pelicon tangential-flow filtration system (Millipore, Harrow, Middx., U.K.) and then harvested by centrifugation at 10000 *g* for 40 min. Typically, a 40-litre culture yield between 100–130 g of cell paste. This was stored at -20 °C until required.

Enzyme purification

All operations were performed either at 4 °C or on ice. All steps after sonication were performed anaerobically under an atmosphere of N_2 unless otherwise stated. *P. aeruginosa* paste (500 g) was suspended in 2.5 litres of 0.1 M-sodium phosphate, pH 7.3, by overhead stirring. Portions (150 ml) of this were sonicated on ice by using a Heat Systems-Ultrasonics (Farmingdale, NY 11735, U.S.A.) sonicator, model W-375, for two 1 min periods at 100 W power. The cell debris was then removed by centrifugation at 10000 *g* for 1 h, leaving the cell-free extract. This was reduced with sodium dithionite, enough to turn Methyl Viologen paper blue.

Solid $(\text{NH}_4)_2\text{SO}_4$ was then added to the cell-free extract, with stirring, until 30% saturation was reached. The precipitate was collected by centrifugation at 10000 *g* for 40 min, and then resuspended in the above phosphate buffer. To this was added an aq. 20% (w/v) solution of sodium deoxycholate to give a final concentration in the protein solution of approx. 2 mg of deoxycholate/mg of protein. This was stirred for 1 h and then centrifuged for 30 min at 20000 *g*.

Between 50 and 75 ml of the soluble deoxycholate extract was stirred into 300 ml of DEAE-cellulose DE52 equilibrated in 50 mM-sodium phosphate, pH 7.3, for 30 min. The following steps were then performed aerobically. The slurry was poured into a column (70 cm $\times$ 5.5 cm) and then washed with the same buffer but containing 100 mM-NaCl. FDH was eluted with 50 mM-sodium phosphate/0.15 M-NaCl/0.05% Triton X-100. Active fractions were pooled, made anaerobic [under 101 kPa (1 atm.) of N_2] and then concentrated on a Minicon filter to between 50 and 100 mg/ml. The enzyme was then stored under N_2 at 4 °C and normally used within 3–7 days.

RESULTS

Purification, stability and properties

FDH in the crude extract is present as an NiR particle complex which was solubilized and dissociated by the addition of sodium deoxycholate. Separation of the NiR and FDH components occurred at the DE52-chromatography stage, although occasionally a residual NiR activity was present in the FDH fraction. In this case the enzymes were separated by an additional $(\text{NH}_4)_2\text{SO}_4$ fractionation, NiR precipitating at about 30% saturation and FDH then precipitating at 80–90% saturation.

Fig. 1. Near-u.v.-visible-region absorption spectra of FDH at room temperature

—, Oxidized; ---, reduced. The enzyme concentration was $13.8 \mu\text{M}$ in 100 mM-Hepes buffer, pH 7.6. The pathlength was 1.00 mm.

We have also used affinity chromatography on phenyl-Sepharose to purify FDH. The enzyme bound tightly to the column and was eluted in buffer containing Triton X-100 (2–10%, w/v). However, although pure enzyme was obtained, the high concentration of detergent necessary to elute the enzyme made subsequent concentration of FDH difficult. Chromatography on DE52 was therefore the preferred method of purification.

Early attempts to purify the enzyme under anaerobic conditions in the absence of sodium dithionite were unsuccessful owing to the introduction of small amounts of air into the system. As a result the FDH activity diminished throughout the preparation. However, the addition of sodium dithionite stabilized the enzyme. This was not due to the scavenging of oxygen from the preparation but was rather due to the reduction of the enzyme. This could be shown by exposing the reduced enzyme to air, or even bubbling with air for 20 min, neither procedure impairing enzyme activity. After this treatment the enzyme did not reoxidize, but was able to reduce an electron acceptor (NBT or potassium ferricyanide) in the absence of formate. After oxidation by this process, activity was still observed when formate was added.

Polyacrylamide-gel electrophoresis on 5% gels showed only three dense bands staining with Coomassie Blue. All of them stained for FDH activity and were of high M_r , indicating polymeric forms of the enzyme. No SDS/polyacrylamide-gel electrophoresis was performed, since material was scarce.

The highest specific activity for the purified enzyme was $774 \mu\text{mol}$ of NBT reduced/min per mg (in sodium phosphate). A preparation from 500 g of cells would yield typically 15–20 mg of protein. Gel filtration of the enzyme on a calibrated Sephacryl S-300 column gave a single species with $k_{av} = 0.234$, corresponding to an apparent M_r of 330 000 for the enzyme in a Triton X-100 micelle. Estimates for haem and molybdenum contents

per mol of enzyme are 2 mol and 1.4 mol respectively. Non-haem iron has not been routinely determined.

The room-temperature absorption spectrum of a preparation lacking NiR activity and with an FDH activity of 760 units (the so-called 'preparation III', see below) is shown in Fig. 1. Both the oxidized and reduced forms are presented. Reduction was accomplished with dithionite. These spectra are very similar to those published by Enoch & Lester (1975). The ϵ values were determined by means of a haemochromogen experiment and are higher than those reported by Enoch & Lester (1975). The α -band at 558 nm is not split, evidence that cytochrome *c* is absent from the preparation.

M.c.d. spectroscopy

An analysis was performed, by using low-temperature m.c.d. spectroscopy, of three different preparations of FDH (*Pseudomonas aeruginosa*) referred to as 'I', 'II' and 'III'. The first, I, has FDH specific activity of 420 units and an NiR activity of $1.8 \mu\text{mol}$ of nitrate reduced/min per mg at 30°C . The NiR activity compares with values of 50 and $10 \mu\text{mol/min}$ per mg for purified enzyme in the so-called 'high-activity' and normal forms (Godfrey *et al.*, 1984). Preparations II and III have no NiR activity and FDH activities of 752 units and 760 units respectively.

Oxidized state

The m.c.d. spectra at 4.2 K and 5 T of the three preparations were recorded over the wavelength range 300–2000 nm (Figs. 2 and 3). The near-i.r. region (800–2000 nm) of the m.c.d. spectra of low-spin ferric haemoproteins, containing protohaem IX, contain bands of characteristic shape arising from porphyrin-to-ferric charge-transfer transitions (Cheng *et al.*, 1973). The energies of these transitions vary according to the nature of the axial ligands which co-ordinate the Fe(III) ion of the haem group. We have calibrated this spectral region with an extensive series of measurements on haems of well-known ligation type employing model compounds as well as proteins of established ligation patterns. Low-spin ferric haems co-ordinated by two histidine ligands give bands with peaks lying in the region 1500–1600 nm, whereas co-ordination by methionine and histidine leads to a broad peak between 1750 and 1850 nm (Foote *et al.*, 1984).

Inspection of Fig. 3 shows that, in all preparations of FDH, there is a major peak at 1545 nm assignable to a bishistidine co-ordinated haem group. In addition there is a weaker peak at 1860 nm, which is due to a methionine-histidine-ligated haem. The presence of the methionine-histidine-co-ordinated haem group is confirmed by the negative feature at 708 nm (Fig. 2). This is the m.c.d. signal arising from the so-called '695 nm' band present in all cytochromes of the *c*-type and taken as diagnostic of methionine-histidine co-ordination (Moore *et al.*, 1985). In preparations I–III the relative intensities of the trough at 708 nm and the peak at 1860 nm decreased together as the methionine-histidine cytochrome is removed. In sample III there is only about 5–10% of this cytochrome remaining, as judged by the intensity of the 1860 nm band. The 708 nm trough is seen only at high gain.

The m.c.d. spectra between 350 and 600 nm, covering the Soret band and α , β -band regions, are consistent with the presence of low-spin ferric haems co-ordinated either by bishistidine or by methionine-histidine. The m.c.d.

Fig. 2. Near-u.v.-visible-region m.c.d. spectra of oxidized FDH at 4.2 K and 5.0 T

(a) Sample I; the enzyme concentration was $63 \mu\text{M}$ in 50 mM-Tris/HCl buffer, p^{H} 8.0, containing 45% (v/v) glycerol; (b) sample II; the enzyme concentration was $85 \mu\text{M}$ in 50 mM-Hepes/NaOH buffer, p^{H} 7.4, containing 50% (v/v) glycerol; (c) sample III; the enzyme concentration was $295 \mu\text{M}$ and $7.5 \mu\text{M}$ in 50 mM-Hepes/NaOH buffer, p^{H} 7.6, containing 50% (v/v) glycerol.

spectra in the Soret and visible region of these two types of cytochrome are very similar to one another and provide no basis for distinction.

We have sought e.p.r. signals due to the low-spin ferric haems in these samples. Only by using a sample of relatively high concentration (0.3 mM), and a temperature below 10 K was it possible to detect a signal at $g = 3.77$ (Fig. 4). This is the g_z component of a rhombic trio of low-spin ferric haem. The g_x and g_y components have not been located. The ramp shape of the g_z signal is similar

to that seen in a number of *b*-type cytochromes of photosynthetic origin (Orme-Johnson *et al.*, 1974) and some model bishistidine-co-ordinated haems (Migata & Iwaizumi, 1981).

This signal is quite typical of a class of e.p.r. spectra known to arise from highly anisotropic low-spin ferric haem that has been the subject of some study and controversy recently (Salerno & Leigh, 1984). Early work led to the suggestion that highly anisotropic low-spin e.p.r. spectra may be diagnostic of amine-histidine or bisamine co-ordination of a haem. However, evidence is now accumulating that certain types of bishistidine co-ordination can bring about such spectra (Salerno, 1984). In particular, the use of imidazole ligands sterically hindered in the 2-position, such as the bis-(2-methylimidazole) complex of protohaem IX, can give rise to e.p.r. spectra having $g_z = 3.53$ with the characteristic ramp shape (Carter *et al.*, 1981). Recently it has been reported that the near-i.r. m.c.d. spectra of such species have a charge-transfer band at $\sim 1500 \text{ nm}$, but with the unexpected feature of an anomalously high intensity. $\Delta\epsilon$ values for bishistidine-ligated haems are $\sim 200 \text{ mol}^{-1}\cdot\text{cm}^{-1}$ at 4.2 K and 5 T. But for model complexes such as bis-(2-methylimidazole)iron-octaethylporphyrin the $\Delta\epsilon$ values rise to $400 \text{ mol}^{-1}\cdot\text{cm}^{-1}$ (Gadsby & Thomson, 1986).

Therefore the presence in FDH of a charge transfer band in the near-i.r. m.c.d. spectrum at 1545 nm with the exceptionally high $\Delta\epsilon$ value of $380 \text{ mol}^{-1}\cdot\text{cm}^{-1}$ and a highly anisotropic low-spin e.p.r. spectrum having a ramp-shaped band at $g_z = 3.77$ leads us to propose that these features arise from a bishistidine co-ordinated protohaem IX having special steric features.

Fig. 3. Near-i.r. m.c.d. spectra of oxidized FDH at 4.2 K and 5.0 T

(a) Sample I; (b) sample II; (c) sample III. Conditions were as in Fig. 2.

Fig. 4. E.p.r. spectrum of oxidized FDH

Sample III conditions were as in Fig. 2. The gain was 1.25×10^6 . Conditions: temperature 5 K; microwave frequency 9.41 GHz; power 20 mW.

Reduced state

The three preparations, I–III, were fully reduced by treatment with dithionite solution. The low-temperature absorption (Fig. 5) and m.c.d. (Fig. 6) spectra have been recorded. The absorption spectra cover the visible region only and, in the low-temperature glass employed, the α - and β -region develops well-resolved vibrational structure. As the *c*-type cytochrome is purified out, the structure in the α, β -region simplifies, leaving a major peak at 556 nm. These changes can also be monitored clearly in the low-temperature m.c.d. spectra (Fig. 6). The α, β -region of the low-temperature m.c.d. spectra of protohaem IX ligated by two histidine ligands and by methionine and histidine contains a very intense, sharp positive and negative band with a crossing point close to the peak in the absorption spectrum (a so-called 'A-term'). In the β -region there are well-resolved, but weaker, features, also positive and negative. It is clear from Fig. 6 that the *b*-type (bishistidine) cytochrome gives rise to the sharp negative and positive bands at 556 and 553 nm respectively. The *c*-type cytochrome has positive and negative features below 550 nm, at 548 and 543 nm. All of these spectral features are temperature-independent in the m.c.d. spectra, showing that they arise from a diamagnetic species, namely low-spin ferrous haem.

There are other features in the low-temperature m.c.d. spectra which are temperature-dependent. All of the broad bands between ~ 560 and 850 nm arise from reduced iron-sulphur clusters (Johnson *et al.*, 1982). In the next section we present a full analysis of the e.p.r. spectra of the reduced state of the three preparations examined here. Signals arising from iron-sulphur clusters of the [4Fe–4S] type can be identified in the dithionite-reduced state of the three preparations. In the oxidized state of the enzyme there are signals at $g = 2.01$ which have the relaxation properties typical of oxidized [3Fe–4S] clusters (Beinert & Thomson, 1983).

The low-temperature m.c.d. spectra of a number of proteins containing reduced [4Fe–4S] and reduced

Fig. 5. Visible-region absorption spectra of reduced FDH at 4.2 K

(a) Sample I; enzyme concentration 150 μM in 30 mM-Tris/HCl buffer, p^H 8.0, containing 70% (v/v) glycerol and 16 mM-sodium dithionite; (b) sample II; enzyme concentration 85 μM in 50 mM-Hepes/NaO²H buffer, p^H 7.4, containing 50% (v/v) glycerol and 4 mM-sodium dithionite; (c) sample III; enzyme concentration 260 μM in 50 mM-Hepes/NaO²H buffer, p^H 7.6, containing 50% (v/v) glycerol and 4 mM-sodium dithionite.

[3Fe–4S] clusters have been reported and their m.c.d. magnetization properties recorded (Johnson *et al.*, 1982). Since the presence of iron-sulphur clusters is clearly established by the detailed e.p.r. studies (see the next subsection), we have sought evidence for their identity in the low-temperature m.c.d. spectra of the reduced-state protein. The reduced states of both the [4Fe–4S] and [3Fe–4S] core clusters have m.c.d. spectra which are relatively broad and somewhat similar in form. The intensities of the reduced [4Fe–4S] signals at 5 T and 1.5 K are $\sim 50 \text{ mol}^{-1} \cdot \text{cm}^{-1}$, and those of the reduced [3Fe–4S] spectra under the same conditions are between 200 and 300 $\text{mol}^{-1} \cdot \text{cm}^{-1}$. It is clear from Fig. 6 that the diamagnetic haem signals between 450 and 550 nm are sufficiently intense to swamp any features due to iron-sulphur clusters. However, in the region 550–850 nm there are broad features which belong to reduced iron-sulphur clusters. Reduced [4Fe–4S] clusters with a spin 1/2 ground state have a negative m.c.d. band between 600 and 650 nm and a more intense positive peak at ~ 740 nm. The m.c.d. magnetization curve gives an intercept value of ~ 0.5 as expected for a spin 1/2 paramagnet with a g -value close to 2.0 (Thomson & Johnson, 1980). The features of the m.c.d. spectrum of a reduced [3Fe–4S] cluster in this wavelength region consist of a negative trough between 550 and 600 nm followed by a positive peak at ~ 700 nm. However, the m.c.d. magnetization curves of both of these features are complex and nested as a function of temperature. This occurs because the ground state is $S = 2$ with a negative, axial zero-field splitting. Thus the magnetization characteristics enable one to determine whether there is any contribution to the spectrum from a [3Fe–4S] cluster in the reduced state.

The m.c.d. magnetization curves measured at 725 nm

Fig. 6. Near-u.v.-visible m.c.d. spectra of reduced FDH at 4.2 K and 5.0 T

(a) Sample I; (b) sample II; (c) sample III. Conditions were as in Fig. 5.

for the preparation I, and at 750 nm for preparation II and III, are shown in Fig. 7. The very steep curve with intercept value of 0.225 obtained for preparation I shows that a significant amount of [3Fe-4S] cluster in the reduced state is contributing to the spectrum. As the amount of 3Fe cluster present decreases in both preparation II and III the curves become less steep. However, even the latter preparation has some contribution from a reduced three-iron cluster, since the magnetization curve deviates from that expected for a spin $1/2$, $g = 2.0$ paramagnet. The theoretical line for such a paramagnet is shown in Fig. 7.

These results agree with those from the e.p.r. studies reported in the next sub-section. By means of e.p.r. it has been possible to monitor the decrease in the intensity of the $g = 2.01$ signal from preparations I-III. This signal is taken to be diagnostic of the presence of an oxidized three-iron cluster.

There is a further feature of interest in the m.c.d. spectra of preparations I and II, namely the strongly-temperature dependent peaks at 440 nm in preparation I and at 440, 419 and 394 nm in preparation II. It was not possible to monitor the m.c.d. spectrum of preparation I to wavelengths shorter than ~ 430 nm because the optical absorbance was too high. The sample concentration was maximized in order to optimize the weak iron-sulphur signals at wavelengths longer than 550 nm. The m.c.d. magnetization characteristics of the peak at 440 nm are reported in Fig. 8. They are quite distinctive and readily recognizable as arising from high-spin ferrous haem with $S = 2$ and a large positive zero-field splitting parameter. This type of nested set has been recorded in this laboratory for a variety of high-spin ferrous haemoproteins of the protohaem IX class

(J. Peterson, A. J. Thomson, A. J. M. Richards & C. Greenwood, unpublished work). Thus we conclude that a proportion of the haem in preparations I and II is in the high-spin ferrous state. However, none is present in preparation III. It will be shown in the next subsection that, under certain conditions, the high-spin ferrous haem may bind NO, presumed to be generated in the nitrate-reduction process, and a three-line e.p.r. signal highly characteristic of certain haem-NO complexes can be detected.

E.p.r.

The three preparations I-III yield e.p.r. signals in the region of $g = 2.0$ in the state as isolated. Fig. 9 displays the spectra recorded at 10 K. In preparation I, a signal at $g = 2.01$ is seen similar in shape to that reported previously for purified NiR (*P. aeruginosa*) (Godfrey *et al.*, 1984) and arising in all probability from an oxidized [3Fe-4S] cluster. As the NiR activity decreases in preparations I-III, this signal is lost to be replaced in preparation III by a sharper signal with a g -value of 2.0. The temperature-dependence of the intensity of these two signals is different. The signal from preparation III persists to 80 K, whereas the intensity of the $g = 2.01$ signal is lost above 20 K. The signal observed in preparation III is similar to that recently reported by Prince *et al.* (1985) to be present in a sample of FDH from *Clostridium pasteurianum* W5. This signal is observed on reduction below a potential of -150 mV, but is absent above this. We have so far carried out no investigations of the dependence of the signal in *P. aeruginosa* FDH on redox potential. Prince *et al.* (1985) attribute the signal to an organic-radical species, possibly a pterin in its anionic semiquinone form.

Fig. 7. M.c.d. magnetization curves for reduced FDH measured in the near-i.r. region

(a) Sample I; the signal intensity was measured at 725 nm at magnetic-field values of 0.61, 1.41, 2.15, 2.87, 3.58, 4.31 and 5.0 T and at temperatures of (*) 1.6 K and (□) 4.2 K. (b) Sample II; the signal intensity was measured at 750 nm at magnetic-field values between 0 and 5.0 T at 1.6 K. Data recorded at 4.2 K and 10.0 K superimpose the 1.6 K curve. (c) Sample III; conditions were as for sample II. Each of the three panels show a calculated m.c.d. magnetization curve (+) for a paramagnet with spin (S) = 1/2 and a g -value of 2.0. (Thomson & Johnson, 1980).

Fig. 8. M.c.d. magnetization curves for reduced FDH, sample II

The signal intensity was measured at 440 nm at magnetic-field values between 0 and 5.0 T at temperatures of 1.6 K, 4.2 K and 10 K.

Fig. 9. E.p.r. spectra of oxidized FDH

(a) Sample I; conditions were as in Fig. 1; the temperature was 10 K and the gain 1.6×10^5 . (b) Sample III; conditions were as in Fig. 1; the temperature was 5 K and the gain 8×10^4 . (c) Sample III; the temperature was 10 K and the gain 5×10^4 . (d) Sample III; the temperature was 20 K and the gain 1.6×10^5 . (e) Sample III; the temperature was 80 K and the gain 5×10^5 . Conditions: microwave frequency 9.41 GHz; power 2.01 mW.

Fig. 10. E.p.r. spectra of reduced FDH

Sample conditions were as in Fig. 5. (a) Sample I; gain 2×10^5 ; (b) sample II; gain 5×10^5 ; (c) sample III; gain 8×10^5 . Conditions: temperature 10 K; microwave frequency 9.41 GHz; power 2.01 mW.

In the dithionite-reduced state of the three preparations (Fig. 10) a complex set of signals which arise from reduced [4Fe-4S] clusters are observed. Previous work established that purified NiR from *P. aeruginosa* (Godfrey *et al.*, 1984) gives rise to a set of e.p.r. signals in the dithionite-reduced state comprising a rhombic trio of g -values: 2.054, 1.952 and 1.878. However, on further reduction of the purified enzyme with excess dithionite, additional peaks developed between the two extreme g -values of the rhombic trio. In a new sample of NiR prepared for the present investigation, e.p.r. studies show the simpler spectrum with g -values slightly different from those reported by us previously (Fig. 11). On warming to 20 K, the rhombic trio of signals are almost totally lost at 2 mW power.

The e.p.r. spectrum at 10 K of sample III is dominated by a species with g -values of 2.044, 1.943 and 1.903 evidently also present in sample II. The temperature-dependence of the intensity of this signal shows that it persists above 30 K at 2 mW power. This signal is not present in the e.p.r. spectrum of NiR from this source (Godfrey *et al.*, 1984) and is therefore attributable to an iron-sulphur cluster of the [4Fe-4S] type associated with FDH. It can be compared with a similar signal obtained from reduced FDH from *Methanobacterium formicicum* having g -values of 2.0465, 1.948 and 1.9111 (Barber *et al.*, 1983).

Fig. 11. E.p.r. spectra of reduced NiR showing the effect of temperature

The enzyme concentration was $200 \mu\text{M}$ in 100 mM-Hepes buffer, pH 7.6. (a) Temperature 10 K; gain 8×10^4 . (b) Temperature 20 K; gain 8×10^4 . (c) Temperature 80 K; gain 2×10^5 . Conditions: microwave frequency 9.41 GHz; power 2.01 mW.

In addition to e.p.r. signals clearly identifiable as arising from reduced [4Fe-4S] clusters, there are others which are particularly obvious at higher temperatures above 20 K, where the intensity of the [4Fe-4S] clusters has diminished. In sample II (Fig. 10) and in the samples of NiR (Fig. 11), there is a three-line spectrum centred at $g = 2.0$ which arises from haem-bound NO in which the odd electron is not coupled to a *trans*-nitrogen atom of a proximal histidine group (Yonetani *et al.*, 1972). The presence of this species was recognized previously in samples of NiR from *E. coli* (Vincent & Bray, 1978). It has not been found in samples of FDH. This may be correlated with the detection, by low-temperature m.c.d. spectroscopy, in samples I and II of some high-spin ferrous haem. Presumably this haem is capable of liganding a gaseous ligand such as NO.

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