Electron-paramagnetic-resonance and magnetic-circular-dichroism studies on the formate dehydrogenase–nitrate reductase particle from *Pseudomonas aeruginosa*

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The membrane-bound respiratory particle complex of *Pseudomonas aeruginosa*, which reduces nitrate to nitrite using formate as the electron donor, was prepared and characterized by e.p.r. and low-temperature magnetic c.d. (m.c.d.) spectroscopy. The particle complex has two enzymic components, namely nitrate reductase (NiR) and formate dehydrogenase (FDH), which are multi-centred proteins containing molybdenum, iron–sulphur clusters and cytochrome. By using results from work on the purified extracted enzymes NiR and FDH to aid in the assignment, it has been possible to observe spectroscopically all the components of the electron-transfer chain in the intact particle. This led to a proposal for the organization of the metal components of the FDH–NiR chain. Molybdenum ions are at opposite ends of the chain and interact with, respectively, the formate–CO₂ couple and the nitrate–nitrite couple. The molybdenum ion at the low-potential end of the chain passes electrons to cytochrome *b* of FDH, a bishistidine-co-ordinated haem with unusual steric restraint at the iron. The next component is a [4Fe–4S] cluster. This comprises all the components of FDH. Electrons are passed to the molybdenum of NiR via a number, probably two, of [4Fe–4S] clusters. No evidence has been found in this work for the presence of a quinone to mediate electron transfer between FDH and NiR. Cytochrome *c* appears to be able to feed electrons into the chain at the level of one of the [4Fe–4S] centres of NiR.

INTRODUCTION

Pseudomonas aeruginosa, when grown anaerobically in the presence of nitrate, forms a membrane-bound respiratory particle complex which reduces nitrate to nitrite by using formate as the electron donor. The particle complex has two enzymic components, nitrate reductase (NiR) and formate dehydrogenase (FDH), which are multi-centred proteins both containing molybdenum, iron–sulphur clusters and haem. The carrier chain appears to be similar to the *Escherichia coli* complex, which is composed of FDH, NiR, quinone and enzyme-bound cytochrome *b* (Enoch & Lester, 1974). *E. coli* FDH and NiR are also multi-centred enzymes, FDH containing molybdenum, iron–sulphur clusters, haem and selenium, and NiR containing molybdenum, iron–sulphur clusters and haem (Enoch & Lester, 1975).

Spectroscopic analysis of these centres has been reported for nitrate reductase from *E. coli* (Vincent & Bray, 1978; Blum & Poole, 1982) and from other sources (Forget & Der Vartanian, 1972; Bosma *et al.*, 1978) and for both NiR and FDH purified from the *P. aeruginosa* complex (Godfrey *et al.*, 1984, 1987). In enzymes isolated from *P. aeruginosa* it was shown that iron–sulphur clusters and molybdenum participate in nitrate reduction, and that haem and iron–sulphur clusters participate in formate oxidation. As an extension to this work we now report an e.p.r. and m.c.d. investigation of the molybdenum, iron–sulphur clusters and haem centres present in the native particle complex of *P. aeruginosa*. This has led us to propose an order of components in the electron-transport chain.

MATERIALS AND METHODS

Organism and culture

Samples of *Pseudomonas aeruginosa* N.C.T.C. 6750 were from the National Collection of Type Cultures, Colindale, London NW9 5HT, U.K. Bacterial culture and harvesting was performed as previously described (Godfrey *et al.*, 1987). Yields were typically between 2.5 and 3.25 g of cell paste/litre of medium, and the cells were stored at –70 °C.

Assay procedures

The activity of the FDH–NiR particle complex was determined by measuring the production of nitrite with formate as the electron donor. The assay mixture contained 0.1 ml of particle complex, 0.1 ml of 100 mM-sodium nitrate and 0.7 ml of 50 mM-sodium phosphate buffer, pH 7.4. The reaction was started by adding 0.1 ml of 200 mM-sodium formate, and the mixture was incubated at 30 °C for 5 min. The reaction was stopped by adding 0.5 ml of sulphuric acid [1% in 25% (v/v) HCl]. Nitrite was then determined colorimetrically using the method of MacGregor *et al.* (1974). One unit of activity is defined as the production of 1 μmol of nitrite/min per mg of protein at 30 °C. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Nitrate reductase and formate dehydrogenase activities, molybdenum, non-haem iron and cytochrome were determined as in the preceding paper (Godfrey *et al.*, 1987).

Preparation of the particle complex and soluble extract

All operations were performed at 2 °C or on ice. A 150 g portion of *Pseudomonas aeruginosa* cell paste was suspended in 1 litre of 100 mM Tris/HCl buffer, pH 8.1. Portions (200 ml) of this were sonicated on ice for 2 × 1 min periods by using a Heat Systems-Ultrasonics (Farmingdale, NY 11735, U.S.A.) sonicator, model W-375. The cell-free extract was isolated by centrifugation at 10000 *g* and the plug discarded. Subsequent steps were then performed anaerobically under an atmosphere of N₂.

Solid (NH₄)₂SO₄ was added to the cell-free extract until 30% saturation was reached. The precipitate was collected by centrifugation at 10000 *g* for 40 min and then resuspended in 100 ml of 100 mM-Tris/HCl, pH 8.1. The soluble and particulate fractions were separated by centrifugation at 50000 *g* for 1 h. The precipitate from this step was homogenized in 20 ml of 100 mM-Hepes buffer, pH 7.5, giving a protein concentration of 33 mg/ml. This sample will be referred to as the 'NiR-FDH particle fraction'.

A 10% solution of Triton X-100 was then added to the particle fraction to a final detergent concentration of approx. 4%, and this was stirred for 20 min. The insoluble material was precipitated by centrifugation for 10 min at 50000 *g*, and then discarded. The supernatant, containing the detergent-soluble proteins, is hereafter referred to as the 'soluble extract'. This fraction was concentrated to between 30 and 40 mg of protein/ml by means of a Minicon filter (B15, Amicon Corp.). Samples were stored under N₂ at 4 °C and used within 4 days of preparation.

Analytical gel filtration

The *M_r* of the particle complex was estimated by gel filtration on a Sepharose 2B-CL column 330 mm long and 25 mm in diameter. The gel was equilibrated in 100 mM-Tris/HCl, pH 8, and then calibrated with alcohol dehydrogenase (*M_r* 150000), catalase (232000), apoferritin (443000) and thyroglobulin (669000). Typically, 1 ml samples were applied (3 mg/ml buffer) and the column eluted at 10 ml/h. Marker proteins were monitored at 280 nm, and the particle was detected by enzyme activity. Elution volume is proportional to log *M_r* (Andrews, 1965), and therefore the *M_r* of the particle could be determined by comparison with a standard curve of marker proteins.

Properties of the particle complex and particle extract

The particle fraction had a specific activity of 0.6 unit/mg of protein, and contained, per mg of protein, 1.23 nmol of haem, 0.485 nmol of molybdenum and 22.7 nmol of iron. The estimated *M_r* of the complex, based on two analytical-gel-filtration experiments, was 668000 ± 12000, assuming a globular conformation for the protein.

The NiR-FDH particulate fraction proved to be of sufficient purity for spectroscopic analysis, but homogeneity was not established by electrophoresis. The metal content is therefore reported per mg of protein rather than on a molar basis.

Owing to the opaque character of the particulate fraction, a transparent soluble extract was prepared for selected m.c.d. experiments by adding Triton X-100. The

addition of detergent disrupted the integrity of the electron-transfer chain from formate to nitrate, but the separate FDH and NiR components remained active.

RESULTS

Fig. 1(a) shows the effect of nitrate on the e.p.r. spectrum of the particle fraction as isolated. The particle is predominantly in the reduced state. Although a small signal at *g* = 2.015 is observable, the predominant species is a reduced [4Fe-4S] cluster exhibiting *g*-values of *g*₁ = 2.045, *g*₂ = 1.945 and *g*₃ = 1.87. This corresponds to one of the reduced [4Fe-4S] clusters of NiR (Godfrey *et al.*, 1984). On addition of nitrate, this signal is progressively reduced in intensity and is replaced at a concentration of 250 μM-nitrate with an intense signal at *g* = 2.015, which can be assigned to an oxidized [3Fe-4S] cluster of NiR. Also present in this spectrum are signals at *g*₁ = 1.983, *g*₂ = 1.976 and *g*₃ = 1.961 arising from the molybdenum centre of NiR. Presented in Fig. 1(b) are the corresponding e.p.r. spectra recorded during the course of formate reduction of the particle fraction. The most obvious effect of formate is the reduction of the [3Fe-4S] cluster of NiR as judged by the disappearance of the *g* = 2.015 signal. The reduction of the [4Fe-4S] cluster of FDH would be expected to give rise to a *g*₃ signal at 1.904, *g*₁ and *g*₂ being indistinguishable from those of NiR (Godfrey *et al.*, 1987). A small signal is generated in this region in the presence of formate. There is, however, no significant increase in intensity in the *g*₁ or *g*₂ region. A number of small changes are also observed at *g* = 1.978 and *g* = 1.93. This might reflect incomplete reduction of the FDH [4Fe-4S] cluster in the presence of formate.

The effect of formate on the e.p.r. spectrum of the oxidized particle fraction is shown in Fig. 2. The particle fraction as isolated is further oxidized by the addition of 350 μM-nitrate. The subsequent addition of 1 mM-formate causes a substantial reduction in the oxidized [3Fe-4S] cluster and the generation of a signal at *g* = 2.0, probably arising from a free-radical species. Also present in this spectrum are signals from a small amount of reduced [4Fe-4S] cluster and Mo(V) of NiR. At higher levels of formate (15 mM) the [4Fe-4S] cluster is further reduced, as is the molybdenum centre. The reduced [4Fe-4S] clusters apparent in this spectrum are predominantly from NiR. However, there is some evidence of reduction of the FDH [4Fe-4S] cluster.

In Fig. 3 the e.p.r. spectrum at 10 K of the dithionite-reduced particle fraction is presented. This shows the reduced [4Fe-4S] cluster from NiR with *g*-values of *g*₁ = 2.045, *g*₂ = 1.945 and *g*₃ = 1.87, but does not reveal the expected reduced [4Fe-4S] cluster from FDH with a *g*₃-value of 1.904. The main difference between this spectrum and the formate-reduced spectrum is the presence of much more intense signals at 1.978 and 1.93. This suggests that these arise from the reduced [4Fe-4S] cluster of FDH. Clearly the form of the spectrum is not the same as that from the reduced [4Fe-4S] clusters of purified FDH (Godfrey *et al.*, 1987). This may imply some degree of spin coupling of the FDH cluster in the particle fraction. It is also noteworthy that the form of e.p.r. spectrum of NiR is not as expected. In a reductive titration of purified NiR, the first signals that are generated are from a reduced [4Fe-4S] cluster with *g*-values of *g*₁ = 2.045, *g*₂ = 1.944 and *g*₃ = 1.87. How-

Fig. 1. E.p.r. spectra of the NiR-FDH particle complex in the presence of (a) sodium nitrate and (b) sodium formate

(a) (i) As isolated; gain 4×10^5 ; (ii) 50 μM -sodium nitrate; gain 4×10^5 ; (iii) 150 μM -sodium nitrate; gain 4×10^5 ; (iv) 250 μM -sodium nitrate; gain 2×10^5 . Conditions: temperature, 10 K; microwave frequency, 9.41 GHz; power, 2.01 mW; modulation intensity, 0.63 mT; protein concentration, 50 μM . (b) (i) As isolated; gain 4×10^5 ; (ii) 5 mM-sodium formate; gain 4×10^5 ; (iii) 20 mM-sodium formate; gain 4×10^5 . Conditions were as for (a).

ever, at higher levels of reductant the spectrum of the reduced [4Fe-4S] clusters broadens considerably and is less well resolved. This suggests spin coupling between two reduced [4Fe-4S] clusters which is absent in the particle fraction. The e.p.r. spectrum of the dithionite-reduced Triton X-100-soluble extract, also shown in Fig. 3, resembles quite closely the spectrum of reduced NiR. The reduced [4Fe-4S] cluster of FDH is also present, although mainly obscured by that of NiR.

The 10 K e.p.r. spectra of the particle fraction in the presence of increasing concentrations of nitrate followed by the addition of 2.5 mM-dithionite is given in Fig. 4(a). In the presence of 150 μM -nitrate and 2.5 mM-dithionite, the particle appears to be fully reduced, as it exhibits the two peaks at $g = 1.944$ and 1.93 observed previously. Also present in this spectrum is a species at approx. $g = 2$ exhibiting a three-line nuclear hyperfine structure. This species is better resolved at 100 K (Fig. 4b), at which temperature the iron-sulphur signals are no longer observed. This species belongs to a ferrous-haem-NO complex, exhibiting a well-resolved nuclear hyperfine coupling to the ^{14}N of the NO. The hyperfine splitting of this species is 1.685 T, similar to the value determined for a number of haem-NO complexes (Nagai *et al.*, 1979). Increasing the concentration of nitrate in the particle fraction to 350 μM before the addition of dithionite has little effect upon the state of

reduction of the iron-sulphur clusters. However, there is an increase in the concentration of the haem-NO species, as evidenced by both the 10 K and 100 K spectra. When the concentration of nitrate added is increased to 750 μM , the [4Fe-4S] clusters are oxidized, as is the [3Fe-4S] cluster of NiR. This is clear from the appearance of a signal at $g = 2.015$ in the 10 K spectrum. Signals from a Mo(V) species, also present in this spectrum, become more apparent in the 100 K spectrum. At 100 K it can be seen that the haem-NO species is still present, but there is also a significant amount of an Mo(V) species. This species exhibits g -values of 1.983, 1.975 and 1.961, which identify this as the molybdenum centre of NiR (Godfrey *et al.*, 1984).

Fig. 5(a) shows m.c.d. spectra, in the visible and near-u.v. region, of the particle fraction as isolated. As expected from the e.p.r. studies, this preparation is at an intermediate state of reduction. The sharp derivative α -peaks at 546 nm and 553 nm are typical of reduced cytochrome *c* and reduced cytochrome *b* respectively. The m.c.d. signals in the Soret region from reduced cytochromes are of low intensity. Thus the derivative-shaped peak at 414 nm is due to an overlap of both oxidized cytochrome *b* and cytochrome *c*. The addition of nitrate to the particle fraction produces marked changes in the m.c.d. spectrum as shown by Fig. 5(b). The increase in the intensity of the Soret band shows

Fig. 2. E.p.r. spectra of the NiR-FDH particle extract showing the effect of sodium formate additions subsequent to nitrate oxidation

(a) 350 μ M-sodium nitrate; gain 2×10^5 ; (b) 350 μ M-sodium nitrate and then 1 mM-sodium formate; gain 2×10^5 ; (c) 350 μ M-sodium nitrate and then 15 mM-sodium formate; gain: 4×10^5 . Conditions were as for Fig. 1(a).

clearly that the amount of oxidized haem has increased by approx. 50%. The α -peak of the reduced cytochrome *b* has completely disappeared, whereas a substantial amount of reduced cytochrome *c* is still observed in the m.c.d. from the α -peak at 546 nm. This observation is in agreement with previous measurements of the rate of reduction of the cytochromes, which suggest that the cytochrome *c* is not in a linear sequence with cytochrome *b*. Thus the cytochrome *c* is slow to respond to oxidation or reduction by nitrate and formate respectively. This suggests that the cytochrome *c* may be on a branch from the formate-nitrate electron-transport chain.

The effect of formate addition to the isolated particle fraction is shown in Fig. 6(a). This shows a decrease in oxidized cytochrome by approx. 25% as evidenced from the decrease in the Soret peak. The increase of reduced cytochrome signal in the α -region appears to be due to an increase in both the cytochrome *b* at 553 nm and the cytochrome *c* at 546 nm. However, it is clear from Fig. 6(a) that formate is unable to reduce fully the particle fraction, which agrees with the e.p.r. results presented above. An almost identical spectrum is obtained for a sample that is fully oxidized with nitrate followed by formate reduction (results not shown). Full reduction of the particle fraction can be achieved with the addition of

Fig. 3. Comparison of reduced e.p.r. spectra

(a) Particle complex as isolated; gain 4×10^5 . (b) Particle complex containing 10 mM-sodium dithionite; gain 4×10^5 . (c) Soluble extract containing 5 mM-sodium dithionite; gain 4×10^5 ; protein concentration 34 μ M. Conditions were as for Fig. 1(a).

dithionite, as shown in Fig. 6(b). The spectrum produced in the presence of excess dithionite shows the presence of reduced cytochrome *c* and cytochrome *b* and a small amount of high-spin ferrous haem, as shown by the peak at 440 nm.

The m.c.d. spectra of the soluble extract in the u.v./visible region are shown in Fig. 7. The oxidized spectrum, Fig. 7(a), is typical of oxidized cytochromes, showing bands throughout this region. Unfortunately, cytochrome *c* and cytochrome *b* overlap and cannot be easily separated. However, a small negative peak at 706 nm is characteristic of many cytochromes *c* from *Pseudomonas* (Foote *et al.*, 1984) and is the counterpart of the so-called '695 nm' band observed in the absorption spectra of cytochrome *c*. It has been proposed that this absorption arises as a result of methionine ligation to haem. The intensity of this band ($\sim 100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) suggests that one cytochrome *c* is present in the soluble extract. The near-i.r. m.c.d. spectrum of the oxidized soluble extract is more informative regarding the type of haem ligation of the two cytochromes (Fig. 8). This shows the presence of two types of haem ligation. The cytochrome *c* exhibits a peak at 1890 nm corresponding to histidine-methionine ligation, whereas the *b*-type cytochrome is at 1560 nm,

Fig. 4. E.p.r. spectra of the NiR-FDH particle extract showing the effect of sodium dithionite additions subsequent to nitrate oxidation

(i) 150 μM -sodium nitrate and then 2.5 mM-sodium dithionite; gain 4×10^5 . (ii) 350 μM -sodium nitrate and then 2.5 mM-sodium dithionite; gain 4×10^5 . (iii) 750 μM -sodium nitrate and then 2.5 mM-sodium dithionite; gain: 2×10^5 . (a) Conditions were as for Fig. 1(a); (b) as (a) except for the temperature, which was 80 K.

Fig. 5. Near-u.v.-visible-region m.c.d. of the NiR-FDH particle complex at 4.2 K and 5.0 T

(a) As isolated; protein concentration 2.75 μM ; containing 50% (v/v) glycerol. (b) In the presence of 10 mM-sodium nitrate; protein concentration 2.25 μM ; containing 50% (v/v) glycerol.

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

(a) Reduced with sodium formate (20 mM); protein concentration $2 \mu\text{M}$; containing 50% (v/v) glycerol. (b) Reduced with sodium dithionite (5 mM); protein concentration $2.75 \mu\text{M}$; containing 50% (v/v) glycerol.

Fig. 7. Near-u.v.-visible-region m.c.d. of the NiR-FDH soluble extract at 4.2 K and 5.0 T

(a) Oxidized with 0.5 mM-potassium ferricyanide; protein concentration $34 \mu\text{M}$; containing 50% (v/v) glycerol. (b) Reduced with 5 mM-sodium dithionite; protein concentration $34 \mu\text{M}$; containing 50% (v/v) glycerol.

suggesting bishistidine ligation. The intensity of these two species indicates that they are present in a 1:1 ratio within the soluble extract. The near i.r. m.c.d. of cytochrome *b* has been shown to be unusually intense for this enzyme ($\Delta\epsilon \approx 400 \text{ M}^{-1} \cdot \text{cm}^{-1}$) compared with cytochrome *c* ($\Delta\epsilon \approx 250 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Gadsby & Thomson, 1986).

The dithionite-reduced soluble-extract m.c.d. is shown in Fig. 7(b). This shows the characteristic sharp derivative peaks at 546 nm and 553 nm associated with cytochrome *c* and cytochrome *b* respectively. This spectrum also exhibits the presence of a considerable concentration of high-spin ferrous haem, showing a

Fig. 8. Near-i.r. m.c.d. of oxidized NiR-FDH soluble extract at 4.2 K and 5.0 T

Conditions were as for Fig. 7(a).

positive peak at 440 nm. Also present is a derivative-shaped peak centred at 581 nm. This appears to be attributable to reduced haem, but is at a longer wavelength than previously observed for reduced cytochrome. A further signal of low intensity ($150 \text{ M}^{-1} \cdot \text{cm}^{-1}$) is observed at 746 nm that is typical of reduced iron-sulphur centres. These are not observed in the particle-fraction m.c.d. because of the low concentrations have to be used in optical experiments with such opalescent solutions.

DISCUSSION

The particle fraction has good activity, showing that the electron-transport chain is intact. This is revealed also by the nitrate oxidation of the iron-sulphur clusters as seen by e.p.r. (Fig. 1a) and also by the oxidation of the haem as seen from the m.c.d. signals (Fig. 5b). However, it appears from Fig. 5(b) that the cytochrome *b* is much more responsive to oxidation than is the cytochrome *c*. The integrity of the electron chain is also shown by the response to formate. Fig. 1(b) reveals that small, but significant, changes in the e.p.r. spectrum are observed on the addition of formate. This is also seen in the m.c.d. spectrum (Fig. 6), where a significant reduction of the haem is achieved by the addition of formate to the particle fraction. Both these Figures do show, however, that formate is unable to reduce fully either the iron-sulphur clusters or the haem. Fig. 2 indicates clearly that formate does donate electrons to the particle after nitrate oxidation. The e.p.r. spectra of the particle in the presence of nitrate and dithionite (Figs. 4a and 4b) demonstrate that nitrate is able to maintain the particle in the oxidized state even in the presence of larger concentrations of dithionite. These additions lead to the generation of a ferrous-haem-NO species that presumably arises from the reduction of nitrate to NO by the particle. This haem species may also be the same one that is observable by m.c.d. as a high-spin ferrous haem in the

dithionite-reduced spectrum, (Fig. 6b). The e.p.r. spectrum of the particle in the presence of high concentrations of nitrate and dithionite ions exhibits an anisotropic signal from Mo(V) that is assigned to the molybdenum centre of nitrate reductase.

The m.c.d. spectra of the soluble extract in the oxidized state show the presence of one cytochrome with methionine-histidine ligation and one with bishistidine co-ordination. The former is typical of covalently bound cytochromes with the same ligation state from *Pseudomonas aeruginosa* and previously studies in this laboratory. They are cytochrome c_{551} and the dihaem cytochrome *c* peroxidases, both of which show near-i.r. m.c.d. bands at 1890 nm and a negative m.c.d. band at 706 nm, assigned as the counterpart of the '695 nm' absorption band. The cytochrome co-ordinated by two histidine ligands gives an m.c.d. band at 1560 nm with a very high intensity ($\Delta\epsilon \sim 400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 4.2 K and 5 T). These spectral features are characteristic of bishistidine-co-ordinated haem with highly anisotropic low-spin e.p.r. spectra, usually giving a low-field component of the *g*-term at $g \sim 3.6\text{--}3.8$ (Gadsby & Thomson, 1986). These properties may arise either because of an unusual orientation of the two histidine ligands or because of large axial distortion field caused by lengthened Fe(III)-histidine bonds generated by the protein fold. We have been unable to detect the e.p.r. signals of this haem in the spectra of the particle or the soluble extract. This is not surprising, since the signals are of low intensity even in purified preparations. This is because the e.p.r. selection rules dictate a vanishingly low transition moment for the limiting case of $g_{\parallel} = 4.0$, $g_{\perp} = 0.0$. We have, however, reported the presence of a signal at $g_{\parallel} = 3.77$ in a purified preparation of FDH (Godfrey *et al.*, 1987).

In the reduced form of the soluble extract the visible region m.c.d. spectra show the features expected from the α -bands of low-spin ferrous cytochromes with methionine-histidine and bishistidine co-ordination. There is also a feature at 581 nm which appears to derive from a low-spin ferrous haem and may be due to a low-spin haem-NO species. The reduced iron-sulphur clusters detectable in the m.c.d. spectrum between 700 and 750 nm are predominantly due to $[4\text{Fe-4S}]^+$ clusters, in contrast with isolated NiR, which contains variable amounts of a $[3\text{Fe-4S}]$ cluster. We therefore suspect that the latter is an artifact generated by extraction of the NiR from the membrane.

The sum of the evidence collected in the present study and those reported in the preceding papers (Gadsby *et al.*, 1987; Godfrey *et al.*, 1987) leads us to propose the organization of the components of the FDH-NiR electron-transport chains shown in Scheme 1.

The evidence is assembled as follows. (1) E.p.r. evidence shows that Mo(V) is contained in both FDH and NiR and, furthermore, suggests that the substrates of each enzyme, formate and nitrate respectively, interact with the molybdenum ion. (2) Cytochrome *b*, having a bishistidine co-ordination, is present at a constant level in purified FDH. There is also one $[4\text{Fe-4S}]$ cluster present, but no $[3\text{Fe-4S}]$ clusters in the most highly purified preparation. The former, designated $[4\text{Fe-4S}]_1$, is characterized by *g*-values of 2.044, 1.943 and 1.903. (3) In less-pure preparations, a second cluster, $[4\text{Fe-4S}]_2$, with *g*-values of 2.044, 1.943 and 1.870, co-purifies with FDH in the absence of the $[4\text{Fe-4S}]$

Scheme 1. Organization of the components of the FDH-NiR electron-transport chains

Abbreviation: Cyt., cytochrome.

centre of NiR which has *g*-values of 2.036, 1.928 and 1.878 (Godfrey *et al.*, 1984) and is designated '[4Fe-4S]₃'. (4) Various amounts of cytochrome *c* (methionine-histidine co-ordination) appear in both NiR and FDH, but in FDH the cytochrome *c* is always associated with [4Fe-4S]₂. (5) The sluggish reduction of cytochrome *c* with formate and also the slow oxidation by nitrate suggests that this cytochrome is not in direct linear sequence but is on a branch of the electron-transport chain. (6) If cytochrome *c* were situated between [4Fe-4S]₁ and [4Fe-4S]₂ we would have expected to see occasionally preparations of FDH containing cytochrome *c* but without [4Fe-4S]₂. This has never been observed in our experiments.

The electron-transport chain proposed has some noteworthy features. The most striking is the presence of molybdenum ion at opposite ends of the chain catalysing different reactions at very different redox potentials. For example, the CO₂,H⁺/HCO₂⁻ couple is -421 mV, whereas the NO₃⁻,2H⁺/NO₂⁻,H₂O potential is +420 mV at pH 7.0. Thus an electron falls through 840 mV in the generation of ATP. It is most unlikely that the ligation sites and co-ordination geometries of the two molybdenum ions are similar in the two enzymes. The e.p.r. *g*-values of the Mo(V) states in both enzymes are different, testifying to this fact.

Cytochrome *b* must have a relatively negative midpoint potential, since it is situated so close to the beginning of the chain. In a bishistidine-co-ordinated haem the potential can be as negative as -300 mV. The positions of the [4Fe-4S] clusters in the chain imply that they all have midpoint potentials more positive than this value. This is unusual for [4Fe-4S]ⁿ⁺ clusters, which redox-cycle between *n* = 2 and *n* = 1. It has been supposed that [2Fe-2S] clusters are involved at more positive potentials. It seems clear that the clusters are in a linear chain and are not involved in feeding electrons into the chain at a lower potential than the Mo of FDH.

There is no quinone shown in Scheme 1 between FDH and NiR. We have been unable to detect quinone in the particle. The activity of the particle is not restored by the

addition of various quinones. If quinone is absent from this chain, it contrasts with that of *E. coli*, in which menaquinone serves to mediate electron transport between FDH and NiR. It is possible, but unlikely, that cytochrome *c* plays this role in *P. aeruginosa*. Reducing equivalents from FAD and NADH could be fed into the chain via cytochrome *c* in order to donate electrons to nitrate in the absence of formate.

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