Purification and properties of a novel cytochrome: flavocytochrome *c* from *Shewanella putrefaciens*

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The major soluble cytochrome isolated from microaerobically grown cells of *Shewanella putrefaciens* has been shown to be a novel type of flavocytochrome with fumarate reductase activity. This flavocytochrome, located in the periplasmic fraction of cell extracts, has been purified to homogeneity and shown to contain 4 mol of haem *c* and 1 mol of non-covalently bound FAD per mol of protein. An M_r value of 63 800 is estimated from sequence analysis assuming 4 mol of haem/mol of protein. In the presence of the artificial electron donor, reduced methyl viologen, the flavocytochrome catalysed the reduction of fumarate but not that of nitrite, dimethylsulphoxide, trimethylamine-*N*-oxide or sulphite. The pH optimum was 7.4 with calculated pK_a values of 6.8 and 8.0 for contributing catalytic groups. The K_m and k_{cat} values for fumarate reduction were 21 μ M and 250 s^{-1} respectively, whereas the corresponding values for succinate oxidation with 2,6-dichlorophenol-indophenol as electron carriers were 200 μ M and 0.07 s^{-1} respectively. Mesoconic acid was a com-

petitive inhibitor of fumarate reduction with a K_i of 2 μ M. Zymogram staining of polyacrylamide gels with purified protein showed a band of fumarate reductase activity. Polyclonal antibodies, raised to the purified flavocytochrome, were shown to titrate out fumarate reductase activity. We conclude that the physiological role of this enzyme is as a fumarate reductase. Optical absorption spectra of the flavocytochrome indicated that all the haems were of the *c*-type and gave α , β and γ peaks at 552.3, 523 and 418 nm in the reduced spectrum with ϵ values of 30.2, 15.9 and 188.2 $mM^{-1}\cdot cm^{-1}$ respectively. Oxidized spectra showed no 695 nm band that would be indicative of His–Met coordination. Two redox potentials were resolved at –220 mV and –320 mV. The cytochrome was reduced by formate in the presence of particulate cell fractions. The relationship of this cytochrome to other low-potential flavocytochromes *c* is discussed.

INTRODUCTION

Shewanella putrefaciens (formerly *Alteromonas putrefaciens*) is a Gram-negative bacterium that is capable of respiration with several electron acceptors including nitrate, fumarate, trimethylamine-*N*-oxide (TMAO), dimethylsulphoxide, Fe(III) and Mn(IV).

Anaerobic respiration with TMAO has been investigated in *Shewanella putrefaciens* because of the role of TMAO in the spoilage of fish. During spoilage TMAO, present in fish tissues, is reduced by *Shewanella* spp. to the organoleptically unpleasant compound trimethylamine (TMA). The electron transport chain from formate to TMAO has been studied in order to identify and characterize the electron carriers involved in the respiratory reduction of TMAO (Morris et al., 1990). The terminal enzyme in this process, TMAO reductase, has been purified and characterized as an 80 000- M_r molybdoprotein (Clarke and Ward, 1987). During anaerobic growth in the presence of TMAO, periplasmic cytochrome c_{552} was co-induced with TMAO reductase (Easter, 1982) and it was later shown that the periplasm contained not one, but several, *c*-type cytochromes (Morris, 1987). The major cytochrome was a flavocytochrome with physicochemical properties different from those of the well-studied phototropic bacterial flavocytochromes and from the *p*-cresol methylhydroxylase of *Pseudomonas putida*.

Fumarate reductases from several Gram-negative bacteria have been studied in detail, including those from *Vibrio* (formerly *Wolinella*) *succinogenes*, *Proteus vulgaris* and *Escherichia coli*

(Kroger, 1980; Cole, 1982; Ackrell et al., 1992). All of these are membrane-bound enzymes which have FAD as the prosthetic group of the catalytic subunit and accept electrons via an iron–sulphur-containing subunit. In *E. coli* the fumarate reductase subunit encoded by the *frdABCD* operon consists of four subunits: FrdA[–] the 69 000- M_r catalytic subunit, FrdB a 27 000- M_r iron–sulphur protein, and two small, but very hydrophobic, subunits of M_r 13 000 and 15 000 respectively that are thought to anchor the complex in the membrane (Lemire et al., 1982). This structure seems to be well-conserved within the enteric bacteria (Unden and Cole, 1983). Soluble fumarate reductases with non-covalently bound FAD have been isolated from some organisms such as *Saccharomyces cerevisiae* (Muratsubaki and Kabane, 1982) and *Desulfovibrio multispirans* (He et al., 1986). Both have a K_m for fumarate of approx. 2.5 mM and are cytoplasmic, with the enzyme from *S. cerevisiae* being a single subunit of M_r 58 800 and that of *Desulfovibrio multispirans* composed of four subunits of native M_r 132 000. Both enzymes are essentially unidirectional. It has been proposed that the lack of succinate dehydrogenase activity in *S. cerevisiae* may ensure a store of succinate under anaerobic conditions when the tricarboxylic acid cycle is not functional.

As the soluble flavocytochrome of *Shewanella putrefaciens* was a major cytochrome induced under anaerobic conditions it was proposed to investigate its properties and physiological role further by purification of the flavocytochrome and study of its properties, including identification of possible electron donors and acceptors. The results of this investigation are reported in this paper.

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; MV, methyl viologen; PMS, phenazine methosulphate; TMAO, trimethylamine-*N*-oxide.
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MATERIALS AND METHODS

Bacterial strain, growth conditions and preparation of cell-free extract

Shewanella putrefaciens strain NCMB 400 was obtained from the culture collection at Torry Research Station, Aberdeen. Bacterial cultures were grown, harvested and a periplasmic fraction was prepared by lysozyme-EDTA treatment as described previously (Easter et al., 1983). The clear red supernatant obtained after centrifugation at 27000 g for 30 min was decanted and retained for study.

Purification procedure

The flavocytochrome was purified by a modification of the procedure of Morris (1987) using $(\text{NH}_4)_2\text{SO}_4$ precipitation as the first step. The pooled 60–100% precipitate was resuspended in 10 mM Tris/HCl, pH 8.4, 5 mM EDTA buffer (TE buffer), dialysed against TE buffer and applied to a 10 cm $\times$ 2 cm column of DEAE-Sephadex CL-6B. The column was developed with an increasing linear gradient of 0–500 mM NaCl in TE buffer (total volume 800 ml). The flavocytochrome eluted at about 160 mM NaCl: fractions having an A_{410} value greater than 0.1 were pooled and analysed for protein and haem content. Fractions containing flavocytochrome, dialysed against TE buffer, were loaded on a 10 cm $\times$ 2 cm column of hydroxyapatite (Bio-Rad HTP DNA grade) equilibrated with TE buffer and eluted with a 1 litre gradient of 1–250 mM K_2HPO_4 in TE buffer, pH 8.4. The flavocytochrome fractions eluted were pooled, dialysed against poly(ethyleneglycol) (PEG) to a suitable volume and then desalted by passage through a 10 cm $\times$ 1 cm column of Sephadex G25 equilibrated with TE buffer.

Amino acid analysis

Amino acid analysis was carried out as described by Gardner (1981) on a Locarte floor model Mark IV amino acid analyser with a 32 cm $\times$ 1 cm column of Locarte resin. Cysteine was determined by cysteic acid after performic acid oxidation of the apocytochrome (Ambler et al., 1969). Tryptophan, largely destroyed by the acid hydrolysis step, was estimated from spectrophotometric measurements at 280 nm using absorption coefficients of $1.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for tyrosine and $5.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for tryptophan (ElKurdi, 1982). Amino acid determinations were averaged from five runs.

PAGE

The method of Laemmli (1970) was used for PAGE in the absence and presence of SDS. Linear gradient gels (7.5–15%) were used for resolving cell fractions and crude preparations. Purified material was electrophoresed on 7.5% or 10% gels. The gels were cast as 1-mm-thick slabs with a 20 cm $\times$ 14 cm resolving gel and a 2.5 cm $\times$ 14 cm stacking gel. Samples for electrophoresis were diluted with the sample buffer of Thomas et al. (1976) and loaded into the sample wells. Electrophoresis was carried out at 10 mA for 1 h, followed by 20 mA until the tracking dye reached 1 cm from the base of the gel (3–6 h). The gels were stained either for protein with Kenacid Blue or for haem by the method of Thomas et al. (1976). The relative molecular mass of the protein was determined by SDS/PAGE with reference to the following marker proteins: horse heart cytochrome *c*, M_r 12400; carbonic anhydrase, M_r 29000; ovalbumin, M_r 45000; BSA, M_r 67000; and phosphorylase b, M_r 97000.

Optical spectra and redox potentiometry

Spectra were recorded using either a Philips SP1800 or an SP8800 recording spectrophotometer. Redox titrations were carried out with a Philips SP1800 recording spectrophotometer equipped with a device for E_h measurement of the cuvette contents. The method was based on that previously published by Dutton (1978). The mediator titrants used were phenazine methosulphate (PMS), phenazine-ethosulphate, diaminodurol, hydroxynaphthoquinone, anthraquinone 2-sulphonate, anthraquinone 2,6-disulphonate and benzyl viologen ($10 \mu\text{M}$). The Pt-Ag/AgCl combination redox electrode (Russell pH Ltd., Auchtermuchty, Fife, Scotland) was calibrated against the 'iron/EDTA' couple (von Schwarzenbach and Heller, 1951; Kolthoff and Auerbach, 1952). An Orion 701 pH/ E_h meter was used to measure the E_h value of the sample.

Haem and flavin determination

Samples of flavocytochrome containing about 70 nmol of haem in a volume of 700 μl were desalted in a 1 cm $\times$ 10 cm column of Sephadex G25 equilibrated with 4.5% formic acid. Samples (1 ml) were collected and their absorbance was measured at 410 nm and 445 nm. Flavin-containing samples were treated with sufficient 2 M K_2HPO_4 , pH 7.0, and water to give a 10 ml preparation containing 0.1 M K_2HPO_4 , pH 7.0. Spectra were recorded over the range 300–500 nm against a buffer blank. Flavin content was determined from the absorbance at 450 nm, assuming an absorption coefficient for FAD of $11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ under these conditions (Dawson et al., 1986). The flavin was identified both by ascending paper chromatography on Whatman No. 1 filter paper using either *s*-butanol/90% formic acid/ H_2O (14:3:3, by vol.) or 1 M K_2HPO_4 as the solvent (Swoboda and Massey, 1965) and also by h.p.l.c. on a 20 μm Aquapore octyl column (250 mm $\times$ 10 mm). Samples for h.p.l.c. (200 μl of a 50 μM solution) were prepared by precipitating the protein with 20% trichloroacetic acid and diluting 100 μl of the flavin-containing supernatant to 0.5 ml with 0.1 M ammonium acetate, pH 7.0. Commercial preparations of FAD and FMN were used as standards.

For haem determination, cytochromes were converted into their pyridine derivatives by the method of Lanyi (1968) by mixing with pyridine/water (1:4, v/v) containing 0.2 M NaOH at a ratio of 1:8 or less, depending on the cytochrome concentration. Haem was measured by the method of Bartsch (1971) using a difference absorption coefficient of $19.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 550 nm for the reduced-oxidized spectrum, or by the method of Wood (1978) using a difference absorption coefficient of $21.7 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for 550 nm where peak heights were measured relative to a baseline drawn between the troughs at 535 and 565 nm.

Enzyme assays

Assays for reduction with fumarate, TMAO, NO_3^- , NO_2^- , SO_3^{2-} or $\text{S}_2\text{O}_3^{2-}$ as electron acceptors were based on the method of Jones and Garland (1977), as described previously (Easter et al., 1983), where the substrate-dependent oxidation of a chemically reduced dye [methyl viologen (MV)] was monitored spectrophotometrically. Nitrate reductase was assayed in MacLeod buffer (10 mM Tris/HCl, 0.3 M NaCl, 50 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM KCl, pH 7.0).

For kinetic studies the buffer was 10 mM Tris/HCl, pH 7.2, containing 0.45 M NaCl. For pK_a studies, pH was varied over the ranges 6.4–9.4 using the Tris buffer and 5.1–7.5 using Mes (50 mM) instead of Tris. Specific activity was expressed as nmol

Table 1 Purification of flavocytochrome *c* from *Shewanella putrefaciens*

Flavocytochrome *c* was purified from a 10 litre culture of microaerophilically grown cells of *Shewanella putrefaciens* as described in the Materials and methods section. At each stage haem content was determined from pyridine haemochrome spectra and fumarate reductase (FR) activity measured from the rate of MV oxidation in the presence of fumarate.

Fraction	Volume (ml)	Haem content (nmol)	Total protein (mg)	FR activity (nmol of MV oxidized/min per mg of protein)	Total activity (nmol/min)	Yield (%)	Purification (-fold)
Periplasm	810	764	175	37.1	6491	100	1
(NH ₄) ₂ SO ₄	34	164	85.4	58.9	5026	77.4	1.59
Ion exchange	39	129	17.1	138	2351	36.2	3.72
Hydroxyapatite	30	90	5.96	224	1337	20.6	6.04

of MV^{•+} oxidized/min per mg of protein, assuming an absorption coefficient of 13 mM⁻¹·cm⁻¹ for MV^{•+} (Thorneley, 1974). Formate dehydrogenase and succinate dehydrogenase activities were assayed spectrophotometrically by the method of Ellis (1959) following the PMS-catalysed reduction of 2,6-dichlorophenol-indophenol (DCPIP) at *A*₆₀₀. Where other electron acceptors were used the assays were identical, except that the alternative electron acceptor replaced the PMS/DCPIP couple. Specific activity was expressed as nmol of redox dye reduced/min per mg of protein.

For kinetic analysis of fumarate reductase and succinate dehydrogenase *K*_m and *V*_{max} values were calculated from non-linear regression analysis.

Antibody production and titration

Antibodies were raised in sheep to the purified flavocytochrome (1 mg) by four intramuscular injections of a protein–Freund's adjuvant emulsion, followed by a booster injection of 0.5 mg of flavocytochrome after 2 weeks. Immunoglobulin G was prepared from sheep antiserum by the method of Steinbruch and Audrun (1969). In titration experiments antibody was preincubated with flavocytochrome for 10 min on ice before assay of fumarate reductase activity.

RESULTS AND DISCUSSION

Purification of flavocytochrome

Flavocytochrome *c* was purified to homogeneity by (NH₄)₂SO₄ precipitation followed by ion-exchange and hydroxyapatite chromatography. As the flavocytochrome was shown to be a fumarate reductase it was possible also to monitor the purification in terms of fumarate reductase activity (Table 1). A purification of 6.0-fold was obtained yielding 6.0 mg of purified flavocytochrome and the flavocytochrome was shown to comprise 20–33% of the total periplasmic *c*-type cytochromes for cells grown microaerophilically with fumarate. The final product had an *A*₄₁₀:*A*₂₈₀ ratio of 3.9 and 20% of the initial fumarate reductase activity was recovered.

The location of the flavocytochrome in the (NH₄)₂SO₄ fractions was confirmed by SDS/PAGE of the precipitates and supernatants from each fraction, followed by silver and haem staining. Haem staining showed the flavocytochrome to be precipitated mostly at 60–80% and 80–100% saturation, although some minor precipitation did occur at lower concentrations. All other periplasmic cytochromes were precipitated at fractions from 0–60%, with the exception of a cytochrome migrating at the gel dye front which co-precipitated with the flavocytochrome at 60–80% saturation. The highest specific activity for fumarate reductase was detected in the 60–80% precipitate, 5-fold higher

than in the 0–60% precipitate and 2.5-fold higher than the 80–100% precipitate.

During ion-exchange chromatography on DEAE-Sepharose the flavocytochrome eluted at about 160 mM NaCl. Comparison of the elution profile with that previously reported for an untreated periplasmic fraction (Morris et al., 1990) showed the removal of the second major cytochrome by (NH₄)₂SO₄ precipitation. This is consistent with its greater hydrophobicity. Both haem staining of polyacrylamide gels of (NH₄)₂SO₄ fractions and the percentage yield values obtained for total haem and flavocytochrome-specific haem showed that (NH₄)₂SO₄ precipitation was effective in removal of most periplasmic cytochromes other than the flavocytochrome, and that the haem content in fractions following chromatography on DEAE-Sepharose was due only to the flavocytochrome. The flavocytochrome eluted from the hydroxyapatite column at about 125 mM K₂HPO₄ and the single peak from gel filtration gave only one band on polyacrylamide gels, indicating purification to homogeneity.

Relative molecular mass, amino acid composition and prosthetic groups

The pyridine haemochrome spectrum was typical of the spectra obtained for *c*-type cytochromes having two thioether bonds between the haem vinyls and the polypeptide chain. The concentration of haem in the standard solution used for spectra and amino acid analysis was 0.102 mM by this method and this gave a value of 4.18 mol of haem per mol of polypeptide based on an *M*_r of 60 548 for the apoprotein, deduced from sequence analysis (Pealing et al., 1992). The flavin content was calculated as 0.83 mol based on an absorption coefficient of 11.3 mM⁻¹·cm⁻¹ at 450 nm (Dawson et al., 1986) and the flavin moiety was identified as FAD both by paper chromatography and h.p.l.c. It was therefore concluded that the holoprotein contains 4 mol of haem *c* and 1 mol of non-covalently bound FAD per mol of polypeptide with a calculated *M*_r of 63 800.

The relative mobilities of flavocytochrome *c* from purified preparations and crude extracts on polyacrylamide gels were in good agreement, and no evidence for subunit structure was found when the protein was treated by boiling in the presence of reducing agents and electrophoresed in the presence of SDS. Determinations of the *M*_r of the purified flavocytochrome by SDS/PAGE on uniform 10% gels indicated an *M*_r value of 68 000 ± 2000 in reasonable agreement with a value of 63 800 deduced from sequence analysis (Pealing et al., 1992); haem proteins are well known for giving anomalous migration rates in polyacrylamide gels. Assuming an integral value of 4 for the haem content, the number of amino acid residues was calculated relative to haem content. The amino acid composition estimated

Figure 1 Electronic absorption spectra of flavocytochrome *c*

Spectra of purified flavocytochrome *c* were recorded on a Phillips SP8800 spectrophotometer. For the reduced spectrum (solid line) sodium dithionite was used as the reductant and for the oxidized spectrum (broken line) the oxidant was air.

Figure 2 Redox titration of flavocytochrome *c*

Nernst plot of the titration of flavocytochrome *c* with dithionite using redox mediators at 10 μ M. The Pt-Ag/AgCl was precalibrated and redox potentials measured on equilibrated solutions after successive additions of sodium dithionite. Peak heights were measured as the difference between A_{552} and A_{563} . The experimental points are denoted by the $\circ$ symbol while the broken line represents the theoretical curve for two equally contributing one-electron redox centres with mid-point potentials of -220 mV and -320 mV.

by amino acid analysis and that from the sequence data (Pealing et al., 1992) are in good agreement; the data confirm that the sequence codes for the flavocytochrome and that the protein is essentially hydrophilic. Also the purity index ($A_{552}:A_{280}$) of 0.945 is consistent with a haem:protein ratio of 4 for a protein of M_r 63800.

Optical spectra

The absolute reduced and oxidized spectra of the cytochrome are shown in Figure 1. The reduced cytochrome had a sharp symmetrical α peak at 552.3 nm, β peak at 523 nm and Soret peak at 418 nm with relative absorption coefficients of 181.2, 95.4 and 1129 $\text{mM}^{-1}\cdot\text{cm}^{-1}$. The Soret peak had a slight shoulder on the short-wavelength side at about 395 nm. The oxidized cytochrome had peaks at 410, 354 and 276 nm. The reduced-oxidized difference spectrum had peaks at 552.3, 523 and 422 nm with troughs at 445 and 405 nm. No 695 nm band, indicative of His-Fe-Met coordination, was observed in the oxidized preparation. The spectra of *Shewanella putrefaciens* flavocytochrome *c* are typical of the Class III cytochromes found in the photosynthetic and sulphate-reducing bacteria (Meyer et al., 1971) and show: (a) a shoulder on the short-wavelength side of the reduced Soret band; (b) a high Soret (red.)/Soret (ox.) ratio (1.55) and (c) no 695 nm band in the oxidized form.

Redox titration

The purified flavocytochrome was titrated with reductant over a wide range of redox potentials; at low redox potentials long equilibration times were necessary. The redox spectrum was typical of a *c*-type cytochrome, but analysis of the titration data (Figure 2) indicated that at least two potentiometrically non-equivalent haems were contributing to the spectrum. The data was resolved by computer analysis into two components with potentials of -220 mV and -320 mV respectively. The results fitted very well to a theoretical curve for two 1-electron components contributing equally to the resultant spectrum. The fitted curve was sensitive to very small changes in estimated mid-point potentials (1–2 mV). Although redox titrations of cytochromes with multiple haems can be difficult to interpret (Pettigrew and Moore, 1987; LeGall and Forget, 1978) these results strongly support the hypothesis of the presence of two equivalent haems at each of these two potentials. There is a possibility of four separate redox potentials, but only if these are in two clusters close to the two values determined

Identification of flavocytochrome *c* as a fumarate reductase

Evidence for the hypothesis that flavocytochrome *c* is a soluble fumarate reductase was obtained in three ways: enzyme and zymogram assay during purification, effect of antibody to purified flavocytochrome *c* on fumarate reductase activity, and kinetic analysis of fumarate reduction. Fumarate reductase activity co-purified at all stages with the flavocytochrome (Table 1) and zymogram staining showed a single band of fumarate reductase activity, following native gel electrophoresis, which corresponded to purified flavocytochrome *c*. In periplasmic extracts a faint additional higher M_r band was sometimes observed on zymogram staining and this was also detected by Western blotting with antiserum to flavocytochrome *c*. Surprisingly, flavocytochrome *c* still retained fumarate reductase activity following SDS/PAGE, although activity was much reduced with only a very faint band observed. This is likely to be due to limited renaturation, as the non-covalently bound FAD essential for enzyme activity is removed by denaturing conditions (Muratsubaki and Katsume, 1985).

The specificity of antiserum against flavocytochrome *c* was determined by Western blotting against purified flavocytochrome and also against total cell lysates of aerobically and micro-aerobically grown *Shewanella putrefaciens*. This blot showed that antibody bound specifically to the flavocytochrome in all samples. The highest density was obtained in extracts from fumarate-

Figure 3 Inhibition of fumarate reductase activity by antiserum to flavocytochrome *c*

Flavocytochrome *c* was preincubated with IgG, purified from antiserum raised to flavocytochrome *c*, for 10 min on ice. Samples were then assayed for fumarate-dependent oxidation of reduced MV.

Table 2 Kinetic properties of flavocytochrome *c*

Fumarate reductase activity was measured from the rate of fumarate-dependent MV oxidation at 600 nm in 10 mM Tris/HCl, pH 7.2, containing 0.45 M NaCl at 25 °C. Succinate dehydrogenase activity was measured from the rate of succinate-dependent DCPIP oxidation at 600 nm under the same conditions.

	Enzyme	
	Fumarate reductase	Succinate dehydrogenase
K_m (μ M)	21 ± 10	200 ± 60
k_{cat} (s^{-1})	250 ± 50	0.07 ± 0.005

grown cells but flavocytochrome was also detected in extracts grown with TMAO or with no exogenous electron acceptor. No band was obtained from cells grown aerobically. The ability of IgG purified from the antiserum to precipitate fumarate reductase activity from periplasmic extracts of *Shewanella putrefaciens* and purified flavocytochrome was investigated (Figure 3). A two-step inhibition was observed for both periplasmic (not shown) and purified protein: the first stage showed a sharp decrease in fumarate reductase activity on preincubation with a relatively small volume (1–20 μ l) of flavocytochrome-specific IgG, followed by a secondary loss of remaining activity on preincubation with larger volumes (up to 400 μ l) of IgG. The inhibition was less acute with purified flavocytochrome *c* than periplasmic samples.

Kinetic analysis of fumarate reduction

In order to study the kinetic properties of the flavocytochrome with fumarate and succinate as substrates, the fumarate reductase and succinate dehydrogenase activities of the flavocytochrome were measured under steady-state conditions. The K_m and k_{cat} values for fumarate and succinate are presented in Table 2. A number of observations led us to conclude that the flavo-

Figure 4 pH-dependence of the catalytic activity of flavocytochrome *c*

The fumarate-dependent oxidation of reduced MV was measured at 600 nm for purified flavocytochrome *c* using 50 mM Tris/HCl buffer (■) or 50 mM Mes buffer (●) in the presence of 0.45 M NaCl over a range of pH values.

cytochrome *c* was a far more efficient fumarate reductase than succinate dehydrogenase. First the K_m for fumarate (21 μ M) was 10-fold less than that for succinate (200 μ M) and secondly the k_{cat} of 250 s^{-1} for fumarate reduction was approx. 3500-fold greater than that for succinate oxidation (0.07 s^{-1}). The soluble fumarate reductases with non-covalently bound FAD, isolated from *S. cerevisiae* and *Desulfovibrio multispirans*, have K_m values for fumarate of approx. 2.5 mM, so are very much less specific for fumarate than flavocytochrome *c*. Like the hydrophilic flavocytochrome *c* of *Shewanella putrefaciens*, these enzymes are essentially unidirectional, catalysing only fumarate reduction. The kinetic data show that flavocytochrome *c* is similar to the fumarate reductases of *E. coli* and *V. succinogenes* in showing a strong specificity for fumarate over succinate. The respective K_m values for the *Shewanella putrefaciens*, *E. coli* and *V. succinogenes* enzymes are 12.6, 17 and 350 μ M for fumarate compared with 200, 1000 and 2000 μ M respectively for succinate (Hirsch et al., 1963; Dickie and Weiner, 1979; Unden et al., 1980). No enzymic activity was detected with any of the other electron acceptors tested despite the similarities between the flavocytochrome and the c_{552} nitrite/hydroxylamine reductase of *E. coli*, and the c_3 cytochromes of *Desulfovibrio gigas* which are reactive with oxidized sulphur compounds.

The pH-dependence of fumarate reduction is shown in Figure 4 from which the pK_a s of two groups involved in catalysis were calculated as 6.8 and 8.0. These groups may both be histidines and by analogy with *E. coli* fumarate reductase, where His-232 appears to be an active-site residue (Schroder et al., 1991), one of these groups could be His-364, the corresponding residue in *Shewanella putrefaciens* fumarate reductase (Pealing et al., 1992). A number of fumarate analogues were tested, both as substrates and inhibitors of the fumarate reductase. None were substrates but mesaconic acid was a strong competitive inhibitor with a K_i of 2 μ M, whereas acrylic acid, transaconitic acid and *trans*- β -hydromuconic acid had no effect. The only structural difference between fumarate and mesaconate is the presence of a methyl group rather than a hydrogen atom. Neither transaconitic acid

nor *trans*- β -hydromuconic acid bound strongly to the active site, implying that these molecules may be sterically occluded.

Structural properties

The structural properties of the flavocytochrome were examined by spectral analysis, redox titrations and PAGE. From optical spectra the fifth and sixth co-ordination positions of the haem irons in *Shewanella putrefaciens* flavocytochrome *c* appear to be provided by histidyl residues, in common with Class III cytochromes. This is consistent with the 10 histidine residues found in the haem domain of the deduced protein sequence and the four CxxCH motifs in this sequence (Pealing et al., 1992). It is normal for a histidine adjacent to the haem-attachment site to be an axial ligand in *c*-type cytochromes. However, evidence from redox titrations is in favour of the hypothesis that these four haems are not in identical environments. Two redox potentials were observed at -220 mV and -320 mV and the transition from full oxidation to full reduction occurred over a span of 120 mV. The low redox potential of the haem centres and the multihaem nature of the protein make the nature of this flavocytochrome very different from soluble *c*-type cytochromes involved in aerobic metabolism and unlike other flavocytochromes analysed previously. The redox potentials of the haems in *Shewanella putrefaciens* flavocytochrome *c* are very different from those in the flavocytochromes of phototrophic bacteria. The *Chromatium* flavocytochromes *c* have redox potentials of about 8 – 28 mV, while those of the *Chlorobium* flavocytochromes *c* are somewhat higher at 98 mV. In each case, the haems and the flavins display similar potentials. The flavin from *Shewanella putrefaciens* flavocytochrome is difficult to titrate spectrophotometrically because the flavin bands are almost entirely masked by the high relative absorption of the multiple haems. The subunit structure of the *Shewanella putrefaciens* flavocytochrome *c* differs from those of the phototrophic flavocytochromes as it consists of a single polypeptide chain rather than separate haem and flavin subunits.

Multihaem *c*-type cytochromes have been shown to have roles in anaerobic metabolism, particularly nitrite reduction and sulphate reduction. For example the anaerobic sulphate reducer *Desulfovibrio vulgaris* contains a tetrahaem cytochrome *c*₃ with bis-His coordination that may act as an electron acceptor from hydrogenase. (Meyer and Kamen, 1982). Cytochrome *c*₃ also has low-redox-potential haems and is of a similar mass (M_r 13000) to the haem domain of flavocytochrome *c*, but is a low-redox-potential cytochrome with one haem titrating at -150 mV and the other two at -230 mV and lacks any flavin group (Feichtner and Kassner, 1979). The nitrite reductase of *E. coli* K12, like flavocytochrome *c*, is a high- M_r haem protein. As originally isolated, the cytochrome was thought to be a hexahaem protein with a relative molecular mass of 69000 (Kajie and Anraku, 1986), but analysis of the coding sequence of the cloned gene shows that it is really a tetrahaem protein (J. A. Cole, personal communication). However, the flavocytochrome *c* described here showed no nitrite reductase activity and no significant sequence similarity (Pealing et al., 1992).

The structural properties of flavocytochrome *c* also differ considerably from those of previously characterized fumarate reductases from other bacteria, including those from *E. coli* and *V. succinogenes*. The enzyme from *E. coli* is composed of four subunits (Dickie and Weiner, 1979; Lemire et al., 1982) and that from *V. succinogenes* of three subunits, such that the complexes are bound to the inner aspect of the cytoplasmic membrane by hydrophobic anchor proteins. The flavin moiety of both enzymes is covalently bound. By contrast the flavocytochrome *c* of

Shewanella putrefaciens is composed of a single subunit with non-covalently bound FAD and is located in the periplasmic fraction. The *Shewanella putrefaciens* enzyme appears to lack an equivalent of the 27000- M_r iron-sulphur subunit found in the *E. coli* complex and differs in the catalytic unit by virtue of a N-terminal haem domain (Pealing et al., 1992).

Physiological function of flavocytochrome *c*

The flavocytochrome was found principally in the periplasmic fraction, with a small amount associated with the membrane fraction and was induced under oxygen-limiting conditions particularly in the presence of fumarate, confirming previous results (Easter et al., 1983; Black, 1991) for the location of *c*-type cytochromes in *Shewanella putrefaciens*. This is consistent with the low hydrophobicity of the protein and with the hypothesis of Wood (1983) who has suggested that all bacterial *c*-type cytochromes are periplasmic proteins. We postulate that the main physiological function of this protein is as a fumarate reductase. The flavocytochrome was not reduced by H_2 , NADH, malate, lactate or formate under anaerobic conditions, but could be reduced by formate in the presence of membrane preparations containing active formate dehydrogenase. It is noteworthy that no low-potential cytochrome *b* has been observed in *Shewanella putrefaciens*. Such cytochromes are typically found associated with formate dehydrogenase in facultative anaerobes where they accept reducing equivalents from formate and conduct electrons to an oxidized redox carrier, leaving protons to be expelled from the enzyme complex to the outer aspect of the inner membrane. The apparent absence of low-potential *b*-type cytochrome associated with formate dehydrogenase in *Shewanella putrefaciens* (Morris, 1987) might suggest the presence of an alternative means of separating high-energy reducing equivalents into protons and electrons in such a manner as to expel protons and retain electrons for further energy conservation steps. Flavocytochrome *c* appears to be a reasonably good candidate for such a role as it is formate-reducible, and the redox potentials of the formate/ CO_2 couple and the flavocytochrome haems are so close that an intermediate component is unlikely (cytochrome b_{FDH} varies in redox potential: -105 mV for *E. coli* and -200 mV for *V. succinogenes*). The flavocytochrome might thus effectively replace the cytochrome *b* usually found associated with other formate dehydrogenase complexes. The presence of flavin in the flavocytochrome may be of particular significance to *Shewanella putrefaciens* with respect to loss of protons during transfer of electrons to the haem group. For a periplasmic flavoprotein accepting reducing equivalents from an internal substrate the protons would be lost to the outside of the coupling membrane, giving net proton translocation.

Conclusions

From our studies we conclude the following: (1) the flavocytochrome *c* from *Shewanella putrefaciens* has an enzymic role as a soluble fumarate reductase; (2) flavocytochrome *c* is a monomeric protein of M_r 63800 containing 4 mol of haem and 1 mol of non-covalently bound FAD per mol of polypeptide; and (3) the environments of the haems are not identical, with redox data showing two centres of -220 mV and -320 mV respectively.

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