Spectroscopic and Kinetic Studies of the Tetraheme Flavocytochrome *c* from *Shewanella putrefaciens* NCIMB400†

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ABSTRACT: Electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) spectroscopic studies were carried out on the tetraheme flavocytochrome *c* from *Shewanella putrefaciens* NCIMB400. The EPR spectrum reveals two sets of *g*-values—$g_z = 2.93$, $g_y = 2.28$, and $g_x = 1.51$; and $g_z = 3.58$—and the MCD spectrum shows a charge-transfer band at 1510 nm. These data combined show that all four hemes are low spin and have a nitrogenous sixth ligand. Sequence comparisons with other tetraheme cytochromes, particularly that from the purple phototroph *H-1-R* [Ambler, R. P. (1991) *Biochim. Biophys. Acta 1058*, 42−47], indicate that the sixth ligands are all histidines. Both the EPR data and the previously reported heme midpoint potentials [−220 and −320 mV; Morris, C. J., Black, A. C., Pealing, S. L., Manson, F. D. C., Chapman, S. K., Reid, G. A., Gibson, D. M., & Ward, F. B. (1994) *Biochem. J. 302*, 587−593] indicate that the hemes fall into two pairs. Stopped-flow kinetic experiments showed that fumarate-dependent heme oxidation was biphasic (k_{cat}[fast] = 400 ± 20 s^{-1}; k_{cat}[slow] = 34 ± 3 s^{-1}), with each phase having the same amplitude, confirming that the hemes are functionally paired.

The tetraheme flavocytochrome *c* from *Shewanella putrefaciens* NCIMB400 is a soluble, unidirectional fumarate reductase (Morris *et al.*, 1994). It has a single polypeptide chain of 571 amino acids (63 800 Da) which appears to form two domains: an *N*-terminal cytochrome domain, containing four *c*-type heme binding motifs (CxxCH), and a flavin domain, which binds FAD[1] non-covalently and is clearly related to the flavin-containing subunits from the family of fumarate reductases and succinate dehydrogenases (Pealing *et al.*, 1992). Other flavocytochromes *c* have been isolated from *Chromatium vinosum* (Bartsch & Kamen, 1960) and *Chlorobium thiosulfatophilum* (Yamanaka *et al.*, 1979) and from *Pseudomonas* species (Koerber *et al.*, 1985), but none of these show any similarity to the flavocytochrome *c* from *S. putrefaciens* 400 (Pealing *et al.*, 1992), and none are fumarate reductases.

The present study focuses on the *N*-terminal cytochrome domain of this flavocytochrome *c* by using EPR and MCD to probe the nature of the axial ligands to the four heme irons. The amino acid sequence of the cytochrome domain is also compared to those of other tetraheme cytochromes. Evidence of paired hemes was also investigated kinetically by the use of stopped-flow measurements.

MATERIALS AND METHODS

Protein Purification. Flavocytochrome *c* was purified from *S. putrefaciens* 400 essentially as described previously (Morris *et al.*, 1994). Bacterial cultures were grown anaerobically and harvested, and the periplasmic fraction was prepared by treatment with lysozyme−EDTA (Easter *et al.*, 1983). The periplasm was incubated with DNase for 30−60 min and then subjected to ammonium sulfate precipitation. The 40−100% ammonium sulfate fraction was resuspended in 10 mM Tris/HCl, pH 8.4, containing 5 mM EDTA and dialyzed before being loaded onto a DE-52 column and developed with a linear gradient of 0−500 mM NaCl. Fractions with fumarate reductase activity were pooled, dialyzed against 10 mM Tris/HCl, applied to a hydroxyapatite column, and eluted with a 0−500 mM gradient of K_2HPO_4 in 10 mM Tris/HCl, pH 8.4. The eluted flavocytochrome *c* fractions were pooled, concentrated, and dialyzed as required. Protein concentrations were determined using the following molar absorption coefficients for reduced flavocytochrome *c*: 30.2, 15.9, and 188.2 mM^{-1} cm^{-1} per heme for the α, β, and γ peaks, respectively (Morris *et al.*, 1994). Since there are four hemes per polypeptide, the extinction coefficients per flavocytochrome *c* are 120.8, 63.6, and 752.8 mM^{-1} cm^{-1}, respectively, for the α, β, and γ peaks. These latter values were previously reported incorrectly (Morris *et al.*, 1994).

EPR Spectroscopy. EPR spectra were recorded using an ER-200D X-band spectrometer (Brüker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow cryostat (ESR-9; Oxford Instruments).

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[1] Abbreviations: bis- His, bis-histidine; His-Lys, histidine−lysine; His-Met, histidine−methionine; CT, charge transfer; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; MCD, magnetic circular dichroism; near-IR, near infrared.

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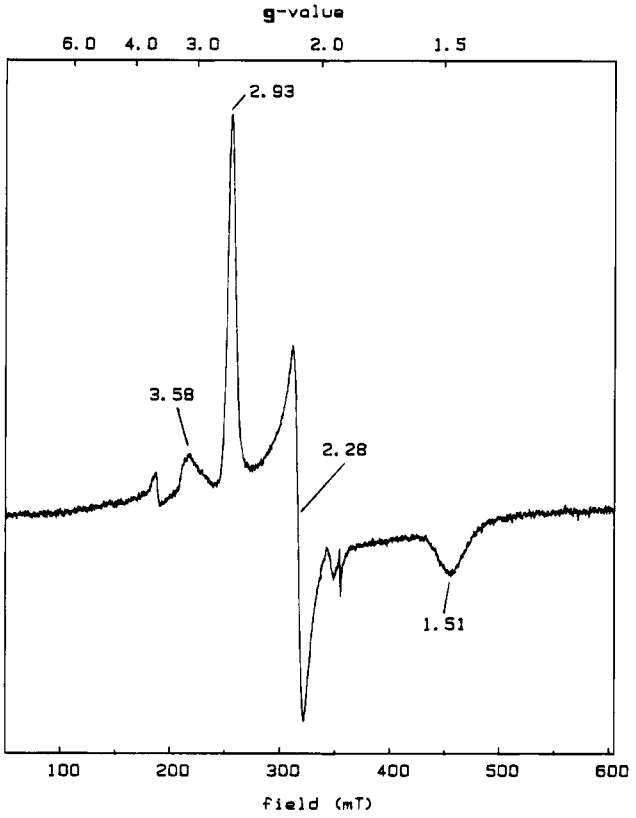

FIGURE 1: X-band EPR spectrum of *Shewanella putrefaciens* flavocytochrome *c*. Total heme concentration was 490 μM in 50 mM MOPS/D_2O buffer, pH* = 7.1. Conditions: temperature, 10 K; microwave frequency, 9.44 GHz; modulation amplitude, 10 G; power, 2.0 mW.

MCD Spectroscopy. MCD spectra were measured using a split-coil superconducting solenoid, type SM-4 (Oxford Instruments), capable of generating a maximum magnetic field of 5T and either a circular dichrograph (JASCO J-500D) for the wavelength range 300−1000 nm or a laboratory-built dichrograph (Gadsby & Thomson, 1990) for the range 800−3000 nm.

Kinetic Measurements. Stopped-flow measurements of fumarate reduction were carried out using an Applied Photophysics SF.17MV stopped-flow spectrofluorimeter. The buffer was 50 mM in HCl adjusted to pH 7.2 with Tris, and the ionic strength was adjusted to 0.5 with NaCl. Stock solutions of dithionite-reduced flavocytochrome *c* (1 μM) and of fumarate (5−100 μM) were prepared in Tris/HCl, pH 7.2 and $I = 0.5$. Fumarate solutions were deoxygenated by degassing under vacuum followed by sparging with oxygen-free nitrogen. The enzyme solution had oxygen-free nitrogen blown over the surface of the solution to prevent denaturation of the protein. The stopped-flow apparatus was washed through with deoxygenated Tris/HCl buffer prior to the experiment. Equal volumes of enzyme solution and fumarate solution (50 μL) were mixed in the stopped-flow apparatus, and the decrease in absorbance of the hemes was followed at 552 nm over a 100-ms time course.

RESULTS

EPR Spectrum. The EPR spectrum of oxidized flavocytochrome *c* at 10 K is shown in Figure 1 and consists of two distinct sets of heme *g*-values. One set of *g*-values comprises $g_z = 2.93$, $g_y = 2.28$ and $g_x = 1.51$, whereas for the other only the g_z-value of 3.58 can be identified. Spin quantitation of these signals revealed that they are of equal intensity; thus each is produced by two of the four hemes of flavocytochrome *c*. The rhombic (V/λ) and axial (Δ/λ) crystal field splitting parameters of the t_{2g} d-subshell have been calculated for the set of *g*-values with $g_z = 2.93$ using the formulae of Taylor (1977). These parameters are shown in Table 1 and are typical of bis-His coordinated cytochromes such as the cytochromes c_3 (Blumberg & Peisach, 1971). The interpretation of the signal produced by the other two hemes ($g_z = 3.58$) is less straightforward since only the g_z value is observed. Also the line shape of this signal has quite a sharp cutoff on the low-field edge, which has been shown by Salerno (1984) to occur when Δ/λ for the heme approaches a limiting value, determined by the ligands, and when V/λ is zero or almost zero. As discussed by Gadsby *et al.* (1989), this makes the unambiguous assignment of the axial ligands impossible solely on the basis of EPR measurements. There is also a derivative-shaped feature at $g = 4.3$ which is almost certainly due to contaminating non-heme high-spin Fe(III).

Table 1: Crystal Field Splitting Parameters for Flavocytochrome *c* Hemes

	g_z	g_y	g_x	V/λ[b]	Δ/λ[b]	Σg_i[c]
Fcc heme type 1	2.93	2.28	1.51	1.87	3.15	16.06
Fcc heme type 2	3.58					
typical Met-His					2.0−2.5[d]	
typical His-His					2.5−3.2[d]	

[a] These values were calculated from the formulae of Taylor (1977). Also shown are the ranges of Δ/λ for different types of axial ligation according to the Truth diagram. [b] *V*, rhombic crystal field parameter; Δ, axial crystal field parameter; λ, spin−orbital coupling constant. [c] Parameter set defined by Taylor (1977), $i = x, y, z$. [d] Approximate range of the axial field values for the two types of axial coordination in the Truth diagram (Blumberg & Peisach, 1971).

MCD Spectrum. The MCD spectrum of oxidized flavocytochrome *c* has been measured in the UV−visible and near-infrared (near-IR) regions. The range 250−750 nm is shown in Figure 2 and is typical of low-spin Fe(III) heme. The lack of any feature between 650 and 750 nm would appear to rule out methionine ligation to any of the four hemes since this has been shown to be accompanied by a band in this region analogous to the 695-nm band in the electronic absorption spectrum of cytochrome *c* (Foote *et al.*, 1984). The electronic absorption spectrum of flavocytochrome *c* also lacks the 695-nm band (Morris *et al.*, 1994).

The near-IR region of the MCD spectrum (Figure 2b) shows a charge-transfer (CT) band at 1510 nm, a region indicative of bis-His axial ligation and ruling out His-Met axial ligation, which would have a CT band in the range 1750−1950 nm (Gadsby & Thomson, 1990). Other nitrogenous ligands can also produce a CT band in the region 1500−1630 nm, e.g., alkaline cytochrome *c*, which has His-Lys coordination (Moore & Pettigrew, 1990), and cytochrome *f*, which has recently been shown from the crystal structure to have the α-amino group of the *N*-terminal tyrosine as its sixth ligand (Martinez *et al.*, 1994).

However, although the g_z-values of amine−histidine-coordinated hemes lie in this range, the line shape remains approximately symmetrical. No well-substantiated examples are known of amine−histidine ligation that give rise to the

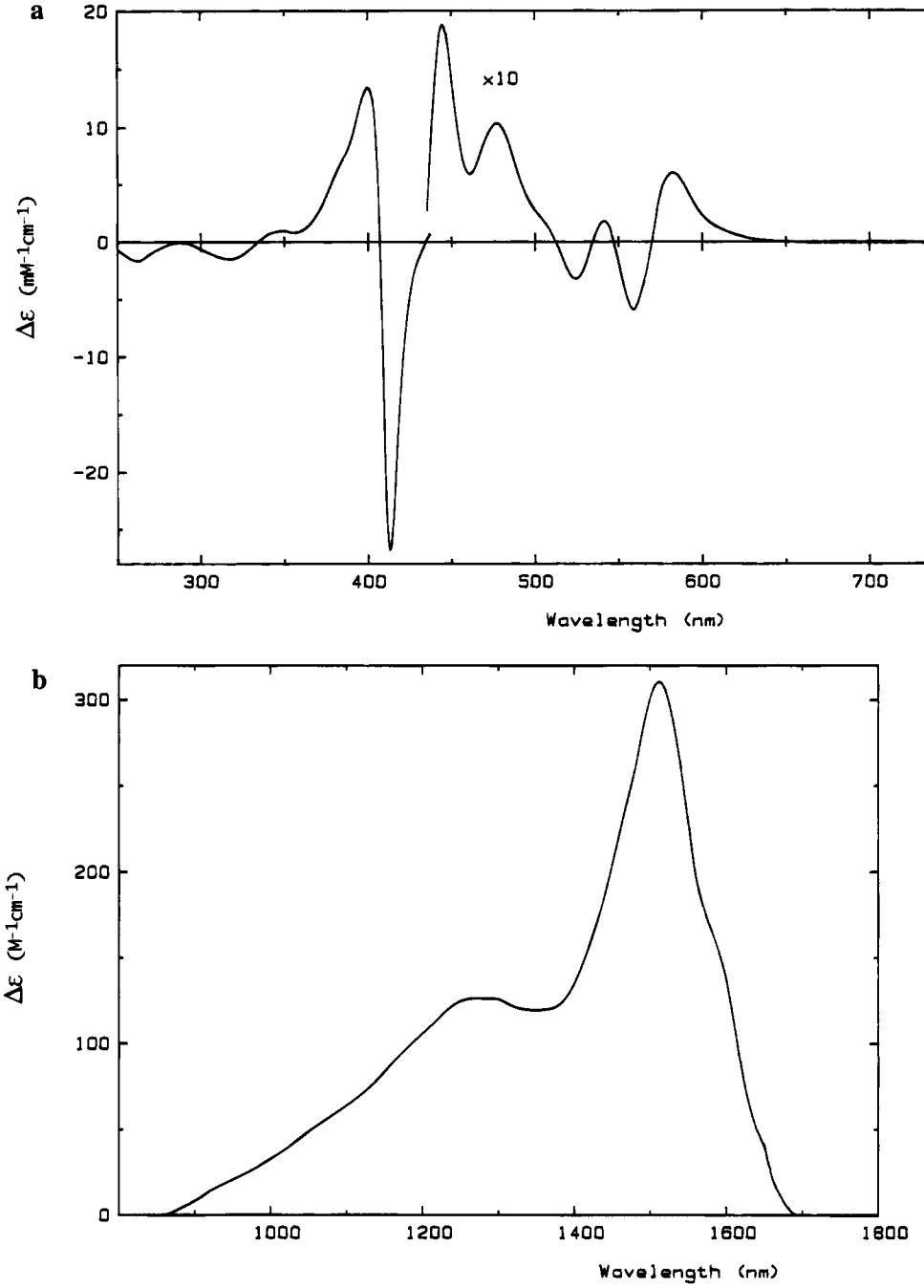

FIGURE 2: MCD spectra of *Shewanella putrefaciens* flavocytochrome *c*. (a) UV−visible MCD spectrum of flavocytochrome *c*. Total heme concentration was 115 μM total heme in 50 mM MOPS−D_2O buffer, pH* = 7.1/glycerol (50%, v/v). Conditions: temperature, 4.2 K; magnetic field, 5 T. (b) near-IR MCD spectrum of flavocytochrome *c*. Total heme concentration was 229 μM total heme in 50 mM MOPS/D_2O buffer, pH* = 7.1/glycerol (50%, v/v). Conditions: temperature, 4.2 K; magnetic field, 5 T.

folded line shape with a low-field cutoff. This suggests, therefore, that the heme ligation is bis-histidine for all four hemes. The differences between the *g*-values of the two sets of hemes reside in the relative orientation of the two histidine planes. The hemes which give rise to the signal at g_z = 3.58 will have histidine planes close to 90° creating an S_4 axis at the iron(III). The intensity of the near-IR CT band in the MCD spectrum due to these hemes is expected to be high with $\Delta\epsilon \sim 400$ M^{-1} cm^{-1} per heme at 4.2 K and 5 T. The remaining two hemes are each expected to have histidine planes closer to an angle of 180°. The near-IR MCD $\Delta\epsilon$ values for each of these hemes will then be $\Delta\epsilon \sim 100-200$ M^{-1} cm^{-1} at 4.2 K and 5 T. Hence the peak intensity for the tetraheme protein is expected to be $\Delta\epsilon > 1000$ M^{-1} cm^{-1} at 4.2 K and 5 T when expressed on the basis of protein molarity. The $\Delta\epsilon$ value determined experimentally at the peak is ~1200 M^{-1} cm^{-1} for the tetraheme protein.

Stopped-Flow Kinetics. Heme oxidation was measured by stopped-flow analysis monitored at 552 nm with the decrease in absorbance due to the hemes followed over a range of fumarate concentrations (2.5−50 μM). The data for each concentration of fumarate fit to a double-exponential curve (Figure 3a), indicating that the reaction was biphasic, having a fast phase with $k_{cat} = 400 \pm 20$ s^{-1} and $K_M = 6 \pm 1$ μM and a slow phase with $k_{cat} = 34 \pm 3$ s^{-1} and $K_M = 2.5 \pm 1$ μM (Figure 3b). Each phase corresponds to half of the total change in absorbance and must therefore be due to the oxidation of two of the four hemes (two electrons).

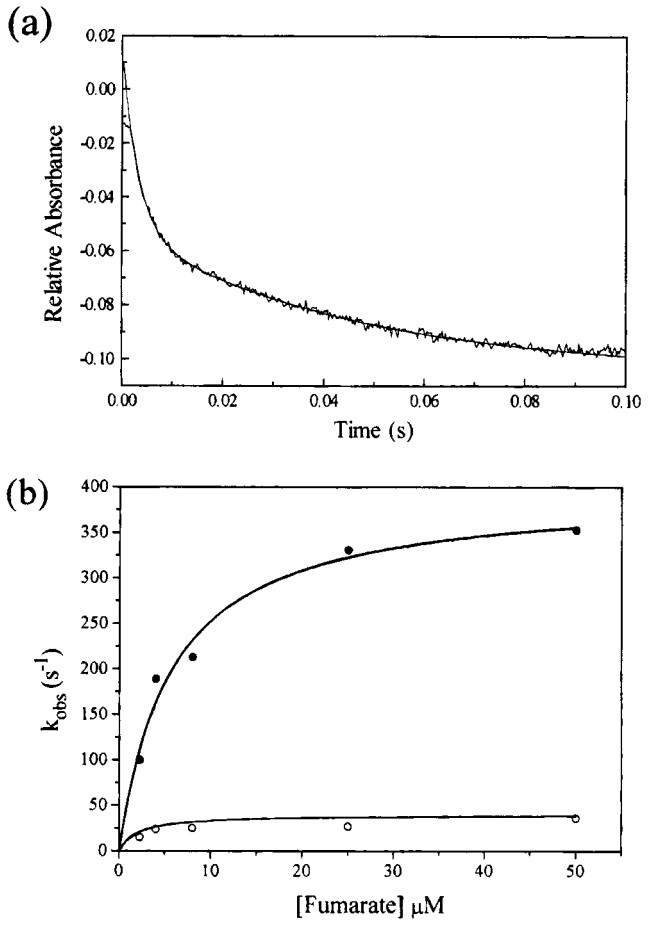

FIGURE 3: Pre-steady-state oxidation of the hemes of flavocytochrome *c*. The oxidation of the hemes was monitored at 552 nm. (a) Example of a stopped-flow trace fitted to a double exponential, indicating biphasic heme oxidation when fumarate is reduced by flavocytochrome *c*. Each phase is half of the total amplitude and so represents two of the four hemes. The initial part of the trace, which does not fit to the double exponential, is the dead time of the equipment. (b) Michaelis–Menten curves of the fast (●) and slow (○) phases of heme reduction. The fast phase is more than 10-fold faster than the slow phase.

DISCUSSION

What Are the Sixth Ligands to the Hemes? The EPR and MCD data for flavocytochrome *c* indicate that not one of the four hemes has methionine as the sixth ligand, and this is backed up by the lack of a methyl peak at about −3 ppm in the NMR spectrum (unpublished data). The EPR data indicate that two of the hemes are typically bis-His ligated. The other two hemes would appear also to have nitrogenous sixth ligands on the basis of the combined data, and the possibilities are histidine, lysine, or, as in the case of cytochrome *f*, the *N*-terminus of the protein. This latter case does not seem to be an option for flavocytochrome *c* since there are two apparently equivalent hemes and obviously only one *N*-terminal amino group.

The sequence of the cytochrome domain of flavocytochrome *c* was examined in order to identify possible candidates for the sixth ligand. Apart from the histidines in the CxxCH heme binding site motifs, there are a further six histidines and six lysines within the first 117 amino acid residues of flavocytochrome *c*, that is, the portion of the sequence which does not align with the flavin subunits of the fumarate reductases and succinate dehydrogenases and is thus assumed to form the cytochrome domain plus any linking polypeptide (Pealing *et al.*, 1992). There are also two methionine residues within this sequence but the spectroscopic data rule these out as ligands. The sequence of the cytochrome domain of flavocytochrome *c* is shown in Figure 4, aligned with the sequence of a tetraheme cytochrome from the purple photosynthetic bacterium *H-1-R* (Ambler, 1991). The sequences are 27% identical including all four of the CxxCH heme binding sites and a further four histidine residues. None of the methionine or lysine residues are conserved between the two sequences. When the cytochrome domain of flavocytochrome *c* is compared to the tetraheme cytochromes c_3 from *Desulfovibrio* species, despite the lack of general sequence similarity, there appears to be an interesting relationship with regard to the axial ligands. Independent crystal structures of two cytochromes c_3 have been determined to high resolution (Pierrot *et al.*, 1982; Higuchi *et al.*, 1984; Picarra-Pereira *et al.*, 1993), and the sixth ligands to the heme irons have been determined from these, showing that all four hemes are bis-His ligated (Figure 5). A simple alignment of the flavocytochrome *c* heme domain and cytochrome c_3 sequences indicates that the ligation pattern of the hemes must be different, because of the nonalignment of putative sixth ligands, if they are considered in order from the *N*-terminus. We have, however, considered the possibility that these two polypeptides are related by circular permutation, a phenomenon that has been observed with other pairs of homologous proteins (Cunningham *et al.*, 1979). If the *C*-terminal half of the cytochrome domain is aligned with the *N*-terminal half of cytochrome c_3 and vice-versa, it appears that all of the sixth ligand histidines of cytochrome c_3 are conserved in position in flavocytochrome *c*, and it therefore seems likely that the ligation patterns are essentially identical. Therefore, on the basis of both the spectroscopic data and the sequence alignments, it seems that the sixth ligands to the hemes of flavocytochrome *c* are all histidines.

Are the Hemes Functionally Paired? Both the EPR data (this work) and the midpoint potentials measured for flavocytochrome *c* (−220 and −320 mV; Morris *et al.*, 1994) indicate that the four hemes of flavocytochrome *c* fall into two pairs. In order to investigate whether this pairing was related to the kinetic properties of the hemes, stopped-flow kinetic measurements were carried out. The oxidation of the hemes gave rise to biphasic traces indicating that they do appear to function as pairs for electron transfer to the flavin. The k_{cat} for the fast phase (see Results) is faster than the k_{cat} for steady-state fumarate reduction (250 ± 50 s^{-1}; Morris *et al.*, 1994), and therefore another stage in the electron-transfer pathway to fumarate must be rate limiting in the full catalytic cycle. The k_{cat} of the slow phase, however, is slower than the steady-state value, indicating that this phase is kinetically irrelevant during turnover when the enzyme acts as a two-electron transferase.

What Is the Physiological Role of the Hemes? The rate of the oxidation of the hemes is dependent on fumarate concentration, and the value of k_{cat} for the fast phase of the oxidation of two of the hemes (k_{cat} = 400 s^{-1}) would appear to indicate that the transfer of electrons from the hemes to the flavin, and subsequently to fumarate, is a physiological reaction. Also since the hemes and flavin are present on the same polypeptide, it seems likely that they are involved in the same electron-transfer pathway. However, the low midpoint potentials of the hemes raises a further problem

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Fcc  1 ADNLAEFHVQNQECDSCHTPDGELSNDSLTYENTQCVSCHGTLAEVAETT 50
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H1R  1 advladmhaemsgcetch.adgapsedg.aheaaacadchggladm.... 44

Fcc 51 KHEHYNAHASHFPGEVACTSCHSAHEK...SMVYCDSCHSFDFNM      92
          .  |.:|   |  :.||.||   ||.    |..  ||.||. : .
H1R 45 ....eaphpahd.gmlectdchmmhedevgsrpacdachddgrta      84
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FIGURE 4: Alignment showing the proposed sixth ligands to the hemes. Sequence alignment of the first 92 residues of flavocytochrome *c* from *S. putrefaciens* (Fcc) with the entire amin -acid sequence of the tetraheme cytochrome from the purple photosynthetic organism *H-1-R* (H1R; Ambler, 1991). The sequences were aligned using the program GAP in the University of Wisconsin Genetics Computer Group (UWGCG) package. Identical residues are indicated by vertical dashes. The heme binding motifs and the four histidines which may be the sixth ligands to the heme irons are shown in bold.

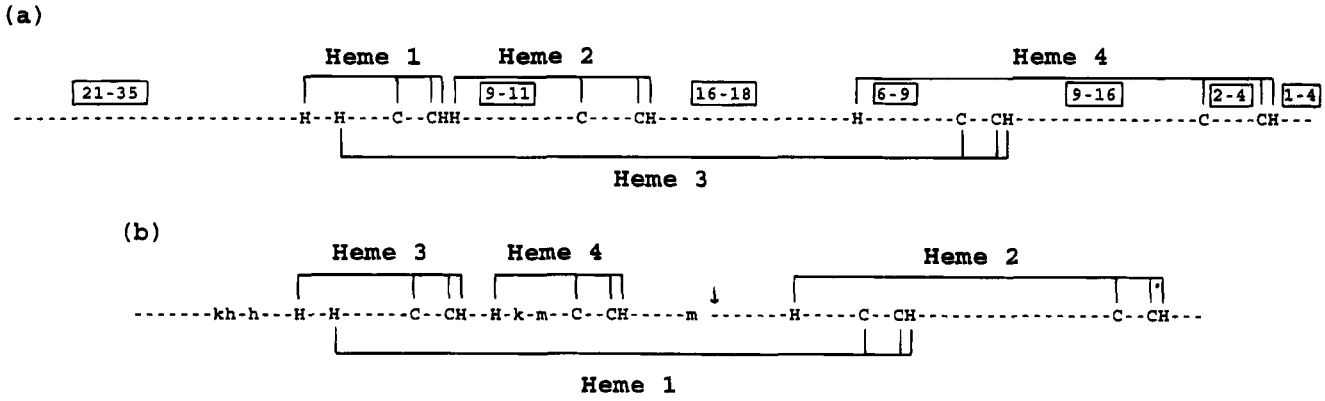

FIGURE 5: Possible relationship between the flavocytochrome *c* cytochrome domain and the cytochromes c_3 from *Desulfovibrio* species. (a) Schematic of the alignment of the six sequences of cytochromes c_3 from *Desulfovibrio* species. The amino acid sequence is represented by the central dashed line. Each dash represents one residue except where the numbers of residues differ between species. In these cases the ranges are indicated by the boxed numbers. The cysteine residues to which each heme group is attached are connected by the solid lines to the two histidine residues that provide the fifth and sixth ligands to the iron of that heme group. The evidence for the iron coordination comes from independent high-resolution crystal structures (Pierrot *et al.*, 1982; Higuchi *et al.*, 1984; Picarra-Pereira *et al.*, 1993). (b) Schematic of the cytochrome domain of flavocytochrome *c* where residues 44−92 and 1−43 have been juxtaposed. A proposed heme ligation scheme based on that of cytochromes c_3 is shown, and the sixth ligand histidines proposed for flavocytochrome *c* are those which are also conserved in the tetraheme cytochrome from *H-1-R* (Figure 4). The other possible ligand candidates (His and Lys residues that are not conserved) are shown in lowercase letters, and residue 1 of the cytochrome domain is indicated by an arrow.

with regard to the identity of the physiological electron donor. This is unknown at present, but there is some evidence to suggest that menaquinone is involved in the pathway. Myers and Myers (1993) showed that a mutant of *S. putrefaciens* MR-1 lacking menaquinone is unable to grow anaerobically with fumarate as the sole electron acceptor. The fumarate reductase was shown to be active by assaying cell extracts for fumarate-dependent benzyl viologen oxidation. This would seem to indicate that menaquinone is a requirement in the electron-transfer pathway to fumarate reductase in *S. putrefaciens*. If this is the case, there must be some modifying factor affecting the potentials of the hemes or of menaquinone such that they are shifted from their midpoint potentials, since menaquinone has a midpoint potential (E_M = −74 mV) much less negative than that of any of the hemes. There are, of course, other examples of enzymes containing redox centers which appear to have anomalous midpoint potentials. For example, the S-2 iron−sulfur center of succinate dehydrogenase appears to have a midpoint potential of −175 mV when the other two iron−sulfur centers are reduced (Cammack *et al.*, 1984; Condon *et al.*, 1985), making transfer of an electron from either of the other iron−sulfur clusters (S-1, E_m = 10 mV; S-3, E_m = 65 mV) or from ubiquinol (E_m = 65 mV) unlikely. However, it has been proposed that interaction between the different iron−sulfur clusters when in the oxidized state alters the redox potential of center S-2 to a more favorable value allowing electron transfer to take place (Salerno, 1991). Other factors such as the binding of the electron donor may also have an effect on the redox potentials of the hemes.

CONCLUSIONS

The conclusions which can be drawn from this work are as follows: (1) All of the hemes of flavocytochrome *c* are bis-His ligated. (2) The four hemes of flavocytochrome *c* are functionally divided into two pairs, allowing the enzyme to function as a two-electron transferase. (3) If menaquinone is the redox partner of flavocytochrome *c*, then there must be some modulation of the redox potentials to allow electron transfer.

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