Tuning of functional heme reduction potentials in *Shewanella* fumarate reductases

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**Abstract**

The fumarate reductases from *S. frigidimarina* NCIMB400 and *S. oneidensis* MR-1 are soluble and monomeric enzymes located in the periplasm of these bacteria. These proteins display two redox active domains, one containing four c-type hemes and another containing FAD at the catalytic site. This arrangement of single-electron redox co-factors leading to multiple-electron active sites is widespread in respiratory enzymes. To investigate the properties that allow a chain of single-electron co-factors to sustain the activity of a multi-electron catalytic site, redox titrations followed by NMR and visible spectroscopies were applied to determine the microscopic thermodynamic parameters of the hemes. The results show that the redox behaviour of these fumarate reductases is similar and dominated by a strong interaction between hemes II and III. This interaction facilitates a sequential transfer of two electrons from the heme domain to FAD via heme IV.

**1. Introduction**

*Shewanella frigidimarina* NCIMB400 and *Shewanella oneidensis* MR-1 are gram-negative, facultative anaerobes belonging to the γ subgroup of Proteobacteria, and exhibit a remarkable respiratory flexibility toward a wide range of terminal electron acceptors, including insoluble metal oxides and soluble compounds such as fumarate [1].

Fumarate respiration is accomplished by a highly efficient protein system, including multiredox-center enzymes that participate in this catalytic process. Under anaerobic conditions and using fumarate as the sole terminal electron acceptor *S. oneidensis* and *S. frigidimarina* produce a large quantity of a periplasmic tetraheme flavocytochrome (fcc3) that acts functionally as a unidirectional terminal fumarate reductase [2]. This enzyme is soluble, monomeric (64 kDa), and its crystal structure reveals that it folds in three domains: the N-terminal heme domain, containing four c-types hemes each axially coordinated by two histidines; the flavin domain which contains a non-covalently bound FAD, located close to the active site; and the clamp domain.

**Keywords:** Respiratory enzyme, Fumarate, Heme, NMR, Redox, Electrostatic interaction
As a consequence, a common mechanism for fumarate reduction in this family of enzymes has been proposed [4,14–18].

In *Shewanella* spp. fumarate reductases the redox centers are very close (≤ 8 Å) and in a quasi-linear spatial arrangement, (see Fig. 1), therefore electron transfer within the tetraheme domain is fast. Electron flow from the heme domain to the FAD must proceed via heme IV, which controls the passage of electrons from the heme domain to the flavin [19]. An array of single electron redox centers leading to a multi-electron active site is found in several respiratory enzymes such as the oxygen oxidoreductases [20], the hydrogenases [21] or the CO dehydrogenases [22].

Although the fumarate reductase from *S. frigidimarina* has been investigated with respect to its biochemical, thermodynamic, kinetic, and mechanistic features [14,16–19,23–28], the microscopic thermodynamic characterization of the individual hemes, including their pairwise interactions, has not been reported. To date, measurements with this level of detail have been restricted to proteins of modest size, typically less than 16 kDa [29–41]. This work is the first example of using NMR techniques for the microscopic thermodynamic analysis of redox proteins of moderate size, opening the way to the detailed characterization of a much wider range of redox proteins. The results presented here for the fumarate reductases from *S. oneidensis* and *S. frigidimarina* allow the proposal of a common functional mechanism for the transfer of two electrons to the FAD active site by a chain of single electron centers.

2. Materials and methods

2.1. Bacterial growth and protein purification

*S. oneidensis* MR-1 and *S. frigidimarina* NCIMB400 cells were grown and the tetraheme flavocytochromes c₃ were purified as previously described in [11] and [23], respectively.

2.2. NMR sample preparation

The protein solutions were exchanged several times into 99.9% ²H₂O using ultrafiltration methods (Amicon; YM-10) thus removing all trace of FAD. Samples with a final protein concentration of 0.5 mM were used. The ionic strength was adjusted to 100 mM by addition of a NaCl solution in ²H₂O. Reduced samples were obtained by the reaction of the

Fig. 1. Structural features of fcc₃ from *S. frigidimarina* and *S. oneidensis* redox centers [3,4]. The spatial disposition of the five redox centers of Sffcc₃ and Sofcc₃ are shown in green and red, respectively. To avoid overcrowding the figure, only the edge-to-edge distances between neighboring centers for Sffcc₃ are shown.

Fig. 2. Region of 2D-EXSY NMR spectra of Sofcc₃ at different oxidation levels at 298 K and pH 7.2. (A) The spectrum shows cross peaks connecting oxidation stages 0–1. (B) the spectrum shows cross peaks connecting oxidation stages 1–3. The lines connect signals for one methyl group of heme II (dashed lines) in different oxidation stages. The Roman and Arabic numbers indicate the heme group and the oxidation stages, respectively.
oxidized protein with gaseous hydrogen in the presence of catalytic amounts of the enzyme hydrogenase (Fe and NiFe isolated from Desulfovibrio vulgaris and Desulfovibrio gigas, respectively). Partially oxidized samples were obtained by flushing the excess hydrogen from reduced samples with argon and then adding controlled amounts of air into the NMR tube with a syringe through the rubber cap as previously described [35]. In the reduced and intermediate stages of oxidation the pH was adjusted inside an anaerobic glove box (Mbraun MB 150 I) with argon atmosphere circulation to avoid the reoxidation of the sample. The pH values reported are direct meter readings without correction for the isotope effect [42].

2.3. NMR spectroscopy

1H-NMR spectra were obtained in a 500 MHz Bruker DRX500 spectrometer equipped with a 5 mm inverse detection probe head with internal B0 gradient coils. All the NMR experiments were carried out at 298 K. Chemical shifts are reported in parts per million (ppm), and spectra were calibrated using the water signal as an internal reference. All values are reported relative to TMS. The program XEASY [43] was used to display the 2D-NMR spectra and assign signals.

2.3.1. NMR of oxidized fcc3 from S. oneidensis

One-dimensional (1D) NMR spectra of fcc3 from S. oneidensis were acquired at pH 7.2 with 128 scans and 64 k data points to cover a sweep width of 32 kHz.

2.3.2. NMR redox titrations of fcc3 from S. frigidimarina and S. oneidensis

In order to establish the complete pattern of oxidation for each heme, 2D-EXSY NMR data sets were collected in the intermediate states of oxidation in the pH range 7.0–8.5. These spectra were acquired with a 25 ms mixing time using and 4096 (t2) × 1024 (t1) data points spanning a sweep width of 32 kHz, with 128 scans per increment. A selective pulse of at least 600 ms was used for water pre-saturation in all experiments.

2.4. Predictions of chemical shifts for the heme methyls

1H paramagnetic chemical shifts of the heme methyls for fcc3 from S. oneidensis were calculated from the empirical equation reported in the literature [44] using the geometry of the heme axial ligands in the crystal structure [3]. 1H diamagnetic chemical shifts of the heme methyls for fcc3 from S. oneidensis were calculated by correcting the heme methyl reference shift (3.48 ppm which is an average of values available for different cytochromes [45]) with the ring current shifts calculated from the crystal structure [3] using a modified version of the software TOTAL [46].

2.5. Redox titrations followed by visible spectroscopy

For both enzymes, anaerobic redox titrations followed by visible spectroscopy were performed as described previously [32]. Protein solutions of ca. 16 μM in 100 mM TRIS/maleate buffer at pH 7.0 and 8.5 were used. For each pH value the redox titrations were performed in both oxidative and reductive directions to check for hysteresis. In order to ensure a good equilibrium between the redox centers and the working electrode [47], a mixture of the following redox mediators was added to the protein solution, all at ca. 2 μM final concentration: anthraquinone-2-7-disulfonate, 2-hydroxy-1-4 naphthoquinone, anthraquinone-2-sulfonate, safranine O, diquat, benzylviologen, neutral red, methylviologen, methylene blue, galloxyanine, indigo tetrasulfonate, indigo trisulfonate and indigo disulfonate. Solution potentials were measured using a combined Pt/Ag/AgCl electrode. Visible spectra were recorded at 298 K in a Shimadzu UV-1203 spectrophotometer, placed inside an anaerobic glove box (Mbraun MB 150 I) under an argon atmosphere, in order to keep the O2 level below 0.5 ppm. The reduced fraction of the fcc3 was obtained using the α band peak located at 552 nm. The optical contribution of the mediators was subtracted by measuring the height of the α peak relative to the straight line connecting the two isosbestic points flanking the α band, as previously described [32,48].

2.6. Thermodynamic modelling

The thermodynamic model used to determine the thermodynamic parameters of both fumarate reductases was described previously [31].

3. Results

The crystal structures of the tetraheme flavocytochromes from S. oneidensis [3] and S. frigidimarina [4] show that all heme iron
The heme methyls A–J are labelled according to their position in the oxidized form. The heme fractions of oxidation, \( x_i \), in each stage of oxidation are calculated according to the equation:

\[
\delta_i = \frac{(\delta - \delta_0)}{(\delta - \delta_0)},
\]

where \( \delta \), \( \delta_0 \), and \( \delta_i \) are the observed chemical shift of the heme methyl in stage i, 0, and 4, respectively [54]. The chemical shifts indicated in parenthesis are assumed values for the diamagnetic reference for heme methyl protons [45].

n.o. — not observed; n.a. — not applicable.

### 3.1. Cross-assignment of Sofcc\(_3\) heme signals to specific hemes in the structure

The first step in the assignment strategy was based on the calculation of the \(^1\)H paramagnetic chemical shifts of each heme methyl group (Table 2). Table 2 shows that the most shifted signal is predicted to be heme methyl 18\(^1\)CH\(_3\) (33.4 ppm), but the experimental data (Table 1) show that two signals, A and B, are located in this region (34.7 ppm and 31.5 ppm, respectively). However, the heme methyl reoxidation patterns (Table 1) show that the patterns of signals A and B are different, indicating that these signals belong to different hemes. The reoxidation pattern of signal B is similar to those of methyl signals C and D, which have chemical shifts of 25.0 ppm and 23.3 ppm, respectively. However, only heme III is predicted to have three heme methyl signals above 24.0 ppm (Table 2), which correlates well with signals B, C and D. Consequently, signal A was assigned with confidence to 18\(^1\)CH\(_3\) and methyls B, C and D to heme III.

### Table 2

<table>
<thead>
<tr>
<th>Heme methyl</th>
<th>Reduced</th>
<th>Oxidized</th>
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<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>2(^1)CH(_3)</td>
<td>3.57</td>
<td>2.37</td>
</tr>
<tr>
<td>7(^1)CH(_3)</td>
<td>3.00</td>
<td>3.59</td>
</tr>
<tr>
<td>12(^1)CH(_3)</td>
<td>3.35</td>
<td>3.58</td>
</tr>
<tr>
<td>18(^1)CH(_3)</td>
<td>3.46</td>
<td>3.57</td>
</tr>
</tbody>
</table>

The heme methyls are numbered according to IUPAC-IUB nomenclature for tetrapyrroles [55].
The remaining heme methyl signals (E–J) include signals from hemes II and IV, but these hemes cannot be discriminated on the basis of the predicted $^1$H chemical shifts of the oxidized protein (Table 2). Nonetheless, the $^1$H diamagnetic chemical shifts predicted for each heme methyl group (Table 2) are quite distinct for hemes II and IV. For heme II, two methyls ($^{18}$CH$_3$ and $^2$CH$_3$) are predicted at 1.97 and 2.37 ppm, respectively, whereas predictions for all heme methyls from heme IV are close to the reference value of 3.48 ppm. In 2D-EXSY NMR

Fig. 4. The pH dependence of the chemical shift of heme methyl group resonances CH$_3$ I, CH$_3$ II, CH$_3$ III and CH$_3$ IV of Sffcc$_3$ (A) and Sofcc$_3$ (B). Squares correspond to stage 1 of oxidation, circles to stage 2, downward-pointing triangles to stage 3, and upward-pointing triangles to stage 4. The solid lines show the result of simultaneous fitting of the NMR and visible data.
spectra of partially oxidized samples two heme methyl signals, E and J show cross-peaks to stage 0 at 1.66 and 2.24 ppm, respectively (Table 1). The pattern of reoxidation of these signals is similar (Table 1), as expected for methyls from the same heme, and their diamagnetic chemical shifts are in agreement with the predicted values for heme II. Therefore, signals E and J were assigned to heme II. Since the oxidation pattern corresponding to the heme signal G was different from those observed for all the other heme signals (Table 1), it was assigned to heme IV. Finally, signal F was also assigned to heme II and signals H and I to heme I since they show similar oxidation patterns to those of signals E and J, and to signal A, respectively.

This assignment strategy allowed four different oxidation patterns to be unambiguously assigned to heme groups in the crystal structure of Sofcc3. Assignment of the signals of Sffcc3 was previously reported [49].

3.2. Thermodynamic characterization of Sffcc3 and Sofcc3

As mentioned above, and as shown in previous works [29,31], the variation of the paramagnetic chemical shifts of only one substituent per heme is needed to map the oxidation patterns of that particular heme. For Sffcc3 and Sofcc3, at least one methyl group from each heme is well shifted from the main envelope of resonances in the oxidized form and thus not subject to signal overlap. The pH dependence of the paramagnetic chemical shifts each heme methyl group and the data obtained for redox titrations followed by visible spectroscopy were fitted to the thermodynamic model described previously [31] to determine the properties of both tetraheme flavocytochromes. The fittings of both NMR and visible data are shown in Figs. 4 and 5. The experimental uncertainty of the NMR data was estimated from the line width of each signal, and the visible data points were given an uncertainty of 3% of the total change in absorbance.

The thermodynamic parameters for Sffcc3 and Sofcc3 are listed in Table 3, and the titration curves of the individual hemes determined from the experimental parameters are shown in Fig. 6.

4. Discussion

The analysis of the thermodynamic parameters obtained for both flavocytochromes (Table 3) shows that the microscopic reduction potentials of the hemes are negative and cover a similar range: −148 to −270 mV and −145 to −286 mV for Sffcc3 and Sofcc3, respectively. The individual reduction potentials are similar with exception of that of heme I which is more positive in Sofcc3 and account for the different shape of the redox titration curves of the two proteins (Fig. 5). In both proteins, heme II has most negative reduction potential and is therefore the heme to become more oxidized in the first oxidation step. The effect of protonation on the heme reduction potentials (redox–Bohr interactions) is small and of similar magnitude for all heme groups in both flavocytochromes (see Table 3 and Fig. 4).

For both proteins, the heme redox interactions (Table 3) are dominated by the interaction between hemes II and III (+65 and +95 mV, for Sffcc3 and Sofcc3, respectively). Fig. 6 shows that the individual titration curves do not display the shape of a Nernst curve and in some cases cross over are observed, an indication that the affinity for electrons of each heme is influenced by the redox state of neighboring hemes. The clearest example of such modulation is observed in the oxidative curve of heme III which shifts towards higher reduction potentials as the oxidation of heme II progresses, due to their very strong negative homo-cooperativity (Fig. 6). Consequently, in the first oxidation stage (loss of the first electron by the tetraheme domain) the reduction potential of heme III is increased so that it remains essentially reduced (cf. the oxidation fraction at 25% for the global protein with those of hemes II and III in Fig. 6).

Fig. 5. Reduced fraction of Sffcc3 (A) and Sofcc3 (B) determined by visible spectroscopy at pH 7.0 (circles) and 8.3 (squares). The solid lines were determined from the simultaneous fitting of the NMR and visible data.

Fig. 6. Oxidized fractions of the individual hemes of Sffcc3 (A) and Sofcc3 (B) at pH 7.2. The curves were calculated as a function of the solution reduction potential using the parameters listed in Table 3. The dashed lines represent the global oxidation fraction.
Table 3
Thermodynamic parameters determined for Sffcc3 (A) and Sofcc3 (B)

<table>
<thead>
<tr>
<th></th>
<th>Heme I</th>
<th>Heme II</th>
<th>Heme III</th>
<th>Heme IV</th>
<th>Ionisable center</th>
</tr>
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<tr>
<td><strong>Energies (meV)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heme I</td>
<td>−228 (5)</td>
<td>23 (3)</td>
<td>3 (3)</td>
<td>−2 (6)</td>
<td>−10 (4)</td>
</tr>
<tr>
<td>Heme II</td>
<td>−270 (5)</td>
<td>65 (2)</td>
<td>0 (3)</td>
<td>−10 (4)</td>
<td></td>
</tr>
<tr>
<td>Heme III</td>
<td>−223 (6)</td>
<td>12 (2)</td>
<td>11 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heme IV</td>
<td>−148 (8)</td>
<td>10 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionisable center</td>
<td>504 (36)</td>
<td></td>
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</table>

Diagonal terms (in bold) represent the oxidation energies of the four hemes and the deprotonating energies for the ionisable center in the fully reduced and protonated protein. Standard errors are given in parenthesis.

The close similarity between Fig. 5A and Fig. 3 of Turner et al. [2] shows that the presence of FAD does not alter the redox properties of the hemes significantly. Indeed, the scale of the interactions measured here together with the structural data summarized in Fig. 1 predicts an anti-cooperative interaction of ca. 10 mV between FAD and heme IV and little or no effect on the other hemes. Comparison with the voltammetric results of Turner et al. [2] is also complicated by the interaction of the redox centers with the electrode surface. However, the FAD is less exposed than the hemes and we may assume that the measured reduction potential, ca. −100 mV at pH 6.0 and −200 mV at pH 8.0, is similar to its value in solution [2]. The redox potential of the FAD is therefore close to those of hemes III and IV, thus ensuring rapid electron transfer.

These observations can be rationalized in functional terms, knowing that the semiquinone state of the FAD is not observed in any of the tetraheme proteins. This interpretation of the data collected for both Sffcc3 and Sofcc3 ensures that the lifetime of a semiquinone, and the possible consequence of producing reactive oxygen species, is minimized. This representation of the data collected for both Shewanella and Polaromonas, and a previous mechanistic proposal [49], are in good agreement with observations made for the membrane-bound fumarate reductase from E. coli, where the authors propose that having the proximal (12Fe–25S) and the medial (14Fe–45S) clusters loaded with electrons ensures that the system is poised for the supply of two electrons to the FAD for catalysis [50].

As previously demonstrated by Schwabl et al. [12], re-reduction of the fcc3 tetraheme domain is mediated by CymA, and heme I of fcc3 is a likely candidate for electron uptake, though all four hemes have some solvent exposure. There is no simple explanation for the difference in mid-point potentials of heme I in fcc3, though it could be related with optimization of electron uptake from the respective forms of CymA since all other hemes show similar redox properties. These include the strong redox interaction between hemes II and III, which is responsible for maintaining a high electron affinity of hemes III and IV in the catalytically relevant redox stages, enhancing the directionality of the intramolecular electron transfer from the heme domain to FAD. The similarity in the properties of Sffcc3 and Sofcc3, is even more striking given the differences between these and small tetraheme proteins that have structures similar to the heme domain of fcc3, and the differences between those proteins [51,52].

In conclusion, this work describes the detailed microscopic thermodynamic characterization of the redox centers, as well their interactions, for two multidox center proteins larger than 64 kDa. This represents a considerable expansion in the range of sizes of redox proteins that have been studied in detail using NMR spectroscopy. It has been shown by Dutton et al. [53] that the distance between the redox centers (below 14 Å) is the most important factor that provides robustness to electron transfer in multi-redox center enzymes. This work addresses the additional requirement of redox potentials that are properly tuned. The data reveal how the thermodynamic properties of the individual hemes, modulated by the oxidation and reduction of their closely spaced neighbors, can favor delivery of electrons to a two-electron active site by an array of single electron cofactors.

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