Elucidation of the Functional Redox Behavior of Fumarate Reductase from *Shewanella frigidimarina* by NMR

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Abstract: NMR spectroscopy has been applied with great success to study electron transfer proteins with multiple redox centers. This study aimed to elucidate the redox behavior the enzyme fumarate reductase from *Shewanella frigidimarina* and particularly to reveal the electron transfer mechanism from the N-terminal domain to the active center. We developed a new strategy encompassing the acquisition of $^1$H-EXSY bidimensional spectra for observation of chemical exchange connectivities in partially oxidized samples of fcc3, estimation of the paramagnetic chemical shifts expected for the heme substituents and their comparison with NMR spectra obtained in the fully oxidized protein. This study allowed obtaining the order of oxidation of the different groups (II-I-III, IV) and gave insights of the functional mechanisms that allow fcc3 to efficiently transfer electrons from the N-terminal domain to the active center.

Resumo: A espectroscopia de RMN tem sido aplicada com grande sucesso no estudo de proteínas de transferência eletrônica, contendo múltiplos centros redox. O objetivo deste estudo consistiu na elucidação do comportamento redox desta enzima, e em particular, na elucidação do mecanismo de transferência dos elétrons desde o domínio N-terminal até ao centro ativo. Foi utilizada uma nova estratégia que englobou a aquisição de espectros bidimensionais $^1$H-EXSY para observação de correlações de permuta química em amostras parcialmente oxidadas de fcc3, estimativas dos desvios paramagnéticos esperados para os substituintes hêmicos e a sua comparação com espectros de RMN obtidos na forma totalmente oxidada. Neste estudo foi possível obter inequivocamente a ordem de oxidação dos diferentes grupos hemo (II-I-III,IV) e elucidar os mecanismos funcionais que permitem à enzima transferir eficazmente os elétrons desde o domínio N-terminal até o centro ativo.

*Shewanella frigidimarina* is a Gram-negative facultative anaerobe bacteria able, in the absence of oxygen, to generate energy from the reduction of several electron acceptors such as Mn(III/IV) and Fe(III) oxides, nitrate and fumarate. As many bacteria, *Shewanella frigidimarina* contains a fumarate reductase that allows it to extract energy for its growth using fumarate as terminal electron acceptor. However, fumarate reductases from *Shewanella* spp. have distinct features when compared with those of other bacteria, such as *Escherichia coli* and *Wolinella succinogenes*. Indeed, these fumarate reductases show bidirectional activity, are multimeric, membrane-bound and contain both FAD and iron-sulfur centres covalently bound to the polypeptide chain. Conversely, the
fumarate reductases isolated from *Shewanella frigidimarina* and *Shewanella oneidensis* (designated as flavocytochromes c\(_3\)), are unidirectional monomeric periplasmatic enzymes with one non-covalently linked FAD and containing no iron-sulfur centres. Instead, they have four c-type heme groups, in a total of five redox centres.

Some structural and biochemical studies have been carried out on the flavocytochromes c\(_3\).\(^6\)-\(^{12}\) The crystal structure of *S. frigidimarina* flavocytochrome c\(_3\) (fcc3) shows that the 571 amino acids polypeptide chain has a three domain arrangement: the N-terminal cytochrome domain, containing four c-type hemes with bis-His axial coordination; the flavin domain, which contains a non-covalently bound FAD; and the clamp domain.\(^6\) To catalyze the fumarate reduction, the FAD at the active site of the enzyme must first receive two electrons and two protons. From the structural data, which shows that heme IV is the closest to FAD (Figure 1), it is expected that electrons are transferred to this centre via heme IV. Electrochemical studies on *S. frigidimarina* flavocytochrome c\(_3\) have shown that electron transfer through the hemes to FAD is fast, which is expected due the close proximity of the redox centres in the protein (Figure 1). However, to understand the electron transfer mechanism and how the electron network within the N-terminal domain is optimised to assure an efficient electron supply to FAD, it is necessary to monitor the redox behaviour of each of the four heme groups in fcc3.

![Figure 1. Structure of flavocytochrome c\(_3\) from *S. frigidimarina*\(^5\) showing the spatial disposition of the five redox centres (hemes I-IV and FAD groups).](image-url)
Several techniques can be used to monitor the redox behaviour of an electron transfer protein with only one redox center. Electrochemical methods such as visible redox titrations and voltammetry are commonly used for such purpose. Although providing a macroscopic description of the redox behaviour of a multiheme protein, these methods do not generally allow the discrimination of individual heme reduction potentials, which in most cases are very similar. Moreover, in multiheme proteins, the redox centres are disposed in very a close proximity, thus affecting the reduction potentials of the neighbouring hemes by redox interactions. Again, a macroscopic analysis is insufficient to discriminate redox interactions between redox centres.

As the number of heme groups increases in an electron transfer protein, several microstates can coexist in solution, which can be grouped according to the number of oxidised hemes in macroscopic oxidation stages linked by successive one-electron reductions. For a multiheme protein, NMR-based approaches have been shown to be the most appropriate to monitor the individual redox behaviour of the heme groups. Most often, NMR can differentiate the signals corresponding to the various heme groups, allowing a self-consistent assignment of the various sets of heme signals to the structure.

Furthermore, in conditions of fast intramolecular electron exchange (between the different microstates within the same oxidation stage) and slow intermolecular electron exchange (between different oxidation stages) on the NMR time scale, the individual heme signals can be discriminated at different degrees of protein oxidation. Since the intrinsic paramagnetic shifts of the heme methyl groups are proportional to the degree of oxidation of that particular heme, these data together with the structural assignment, can provide information on the relative order of oxidation of the hemes. Typically, this can be achieved by following the position of methyl signals, which are shifted towards low field, outside the protein envelope, by 2D-EXSY NMR redox titrations.

However, due to the large size of fcc3, challenging difficulties must be overcome. Although fcc3 heme groups are low spin in both the reduced and oxidised states of the protein, the slow tumbling observed for this protein impairs the acquisition of 2D-NMR spectra of sufficient quality in the fully-reduced or -oxidised protein, preventing the assignment of the heme signals within the structure. To overcome this difficulty, a fcc3 mutant was produced by replacing the axial ligand of heme IV His61 by one Ala residue, according to the procedure described by Rothery and co-workers. Comparison between the fcc3 and fcc3 mutated protein was carried out by 1D-1H-NMR spectra and 2D-1H-EXSY. The information obtained, together with the chemical shifts predictions calculated from the fcc3 structure, allowed us to attribute the heme methyl NMR signals to each heme. This information was then used to follow each heme methyl signal through the different oxidation degrees of the protein.

Samples of partially oxidized fcc3 were prepared in 99.9% 2H2O as described in the literature. 2D-EXSY NMR experiments with 25 ms mixing time were acquired with protein samples at different oxidation levels. The percentage of oxidation of each heme group
was calculated from the total paramagnetic shift observed for the corresponding methyl group at the five oxidation stages (Table 1). These data clearly show that the four hemes of fcc3 can be grouped in pairs, according to their redox behavior. In fact, heme I and II are more oxidized in latter oxidation stages (oxidation stages 1 and 2), whereas heme III and IV are essentially reduced.

Table 1. Heme oxidation percentages of *S. frigidimarina* flavocytochrome *c* 3 in each of the five stages of oxidation. The nomenclature of the heme groups is based on the order of attachment to the CXXCH motifs in the amino acid sequence.

<table>
<thead>
<tr>
<th>Oxidation stage</th>
<th>Oxidation fraction</th>
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<tbody>
<tr>
<td></td>
<td>HEME I</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
</tr>
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*Since hemes III and IV are mainly reduced at the first oxidation stages, the corresponding heme methyl NMR signals fall in very crowded regions of the NMR spectrum preventing their assignment. Thus, the values indicated were estimated from the remaining oxidation percentages.

Concerning the electron transfer mechanism that assures an efficient supply of electrons towards fumarate reduction, our results are of extremely functional relevance. In fact, it is clear that in the fully reduced protein, electrons can be transferred from heme III and IV to FAD. However, at oxidation stage 2, where the protein is depleted in two electrons, the results showed that hemes III and IV are mainly reduced (Table 1). When transferring two electrons to the active site, hemes III and IV can be promptly electron refilled by oxidation of hemes I and II. This ensures that hemes III and IV are kept reduced until later in the oxidation, optimizing the availability of electrons to be transferred to FAD.

The results obtained in this study clearly show that the individual heme groups at the fcc3 N-terminal domain behave differently, forming a concerted network that ensures a highly efficient two-electron transfer to the FAD-mediated fumarate reduction.

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**References**


