

Electron transport in sulfate-reducing bacteria

Molecular modeling and NMR studies of the rubredoxin – tetraheme-cytochrome- c_3 complex

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A hypothetical model of the complex formed between the iron-sulfur protein rubredoxin and the tetraheme cytochrome c_3 from the sulfate-reducing bacteria *Desulfovibrio vulgaris* (Hildenborough) has been proposed utilizing computer graphic modeling, computational methods and NMR spectroscopy. The proposed complex appears feasible on the basis of complementary electrostatic interaction and steric factors and is consistent with the data from NMR experiments. In this model, the non-heme iron atom of rubredoxin is in close proximity to heme 1 of cytochrome c_3 . The complex is stabilized by charge-pair interactions and hydrogen bonds. This complex is compared to the flavodoxin-cytochrome c_3 complex previously proposed [Stewart, D. E., LeGall, J., Moura, I., Moura, J. J. G., Peck, H. D. Jr, Xavier, A. V., Weiner, P. K. & Wampler, J. E. (1988) *Biochemistry* 27, 2444–2450] and new NMR data shows that both proteins interact with the same heme group of the cytochrome as postulated.

The proteins involved in electron transfer in the sulfate-reducing bacteria (*Desulfovibrio* sp.) have been well characterized regarding spectral properties, cellular localization, sequence, redox center composition and function [1]. In addition, the structures of several of these proteins have been determined crystallographically: the small non-heme iron-containing protein rubredoxin [2–5], the tetraheme cytochrome c_3 [6, 7] and the flavoprotein flavodoxin [8].

One of the most interesting of these proteins is the tetraheme cytochrome c_3 ($M_r = 13\,000$) which has structural and redox properties distinctly different from the monoheme cytochromes c . The hemes of the cytochromes c_3 exhibit separate and distinct redox properties with negative redox potentials as low as -340 mV [9]. In the two known crystal structures [6, 10] the hemes are well separated (average Fe–Fe distance of 1.45 nm) and are arranged with a particularly obvious lack of planar alignment (average heme–heme angle of 72°). As described previously [11], the surface charge and exposure of each of these hemes at the corresponding face of the protein is unique. Spectroscopic and kinetic methods have been employed to investigate the interactions between several of these redox proteins: hydrogenase with cytochrome c_3 , rubredoxin, ferredoxin and flavodoxin [12]; ferredoxin with cytochrome c_3 [13–18], for which a molecular model of the complex has recently been proposed [19]; cytochrome c_3 with flavodoxin and rubredoxin [20]. We have recently proposed a model of the flavodoxin-cytochrome c_3 complex [11] and this study includes a similar investigation of the interaction of cytochrome c_3 with rubredoxin. In addition, support for both models is presented from detailed NMR studies.

Computer graphics have been successfully utilized in the study of the interactions of several different types of proteins

[21–26]. In most of these studies, computer graphics models provided a method to explain the results from physico-chemical studies and served to direct further investigations. Both roles are illustrated here. A common scheme for interactions between c -type cytochromes and their redox partners is that the heme crevice, surrounded by positively charged lysine residues, interacts with acidic residues surrounding the redox partner's functionally active group. This has been encountered in complexes between cytochrome c and a variety of proteins: cytochrome- c peroxidase [22] cytochrome- c oxidase [27], flavodoxin [28, 29] beef mitochondrial reductase [30] cytochrome b_5 [21, 26] and the cytochrome bc_1 complex [31]. Although the structural features and redox potentials of cytochrome c_3 are distinctly different, it might well follow the same pattern, since its heme crevices are surrounded by lysine residues and its redox partners are quite acidic. This then suggests a simple approach for predicting interaction sites for cytochrome c_3 with its redox partners through model building: to search for sites on the redox partner which are maximally complementary to the ring of lysine residues on the cytochrome and which offer minimal steric conflicts.

For these studies, we have chosen to investigate the interaction between cytochrome c_3 , flavodoxin and rubredoxin from *D. vulgaris* (Hildenborough) since three-dimensional structures are available, thereby allowing a molecular modeling interpretation of the results from the NMR experiments. Complementary NMR studies are an important addition to this type of study providing experimental support for the computational approach.

Physiological and molecular biological observations indicate that cytochrome c_3 and rubredoxin are localized in different cellular compartments, the periplasm and cytoplasm, respectively [32, 33]. The complex formed between these two proteins may not be of direct physiological significance due to

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their compartmentalization, but they provide excellent models for the study of electron transfer between different redox centers, as similarly observed for other systems [11, 34]. A more sophisticated model for the *in vivo* interaction might envisage a transmembrane complex of electron-transfer proteins of the localization of the tetraheme cytochrome c_3 in both the periplasm and cytoplasm. However, *in vivo* studies have shown that cytochrome c_3 is necessary for a rapid reduction of rubredoxin by hydrogenase, thus rubredoxin and cytochrome c_3 can function as redox partners [12]. We anticipate that these modeling studies will aid interpretation of the *in vivo* experiments and the assignment of the heme methyl resonances in the NMR spectra to specific heme groups. Thus, these models should also prove useful in elucidating interactions between cytochrome c_3 and its other redox partners such as ferredoxin and hydrogenase [35].

METHODS

Molecular modeling

Model building was performed on an Evans & Sutherland PS340 using the molecular graphics program MOGLI (Evans and Sutherland Computer Corporation, Salt Lake City, Utah 1985). The electrostatic surfaces and fields were calculated and displayed using AMBER [36] and associated programs [23, 37] as indicated in [11]. A +1 kcal/mol (4.184 kJ/mol) charge was used for the iron atoms. The charges assigned to all other heme atoms are those from [38]. Computations were performed on a Digital Equipment Corporation VAX 11/780 running VMS v. 4.4. Surface exposures of the hemes were calculated using the programs MS [37].

The coordinates for *D. vulgaris* (Miyazaki) cytochrome c_3 at 0.18 nm resolution [6] and *D. vulgaris* (Hildenborough) rubredoxin at 0.15 nm resolution [3] were obtained from the Brookhaven Data Bank [39].

Rubredoxin was docked to each exposed heme of cytochrome c_3 . Each docked structure was manipulated in order to maximize the interaction between the acidic groups of rubredoxin and the lysine residues surrounding the heme crevices of cytochrome c_3 , and to eliminate steric conflicts.

NMR

D. vulgaris (Hildenborough) cytochrome c_3 , flavodoxin and rubredoxin were purified by previously reported methods [40–42]. The proteins were extensively dialyzed against distilled water at 4°C then lyophilized three times from D₂O. The nominal pH of solutions was adjusted to a similar value with NaOD or DCl. In order to assure that the perturbation induced in chemical shift and/or line broadening were not due to pH effects, the samples were routinely dialyzed in separate bags in the same vessel against D₂O. Protein concentrations were determined from previously reported molar extinction coefficients [40–42]. Aliquots of the interacting protein (rubredoxin) were added to the NMR tube containing cytochrome c_3 up to a rubredoxin/cytochrome c_3 molar ratio above the stoichiometric amount. NMR spectra were recorded after each individual addition on a CXP-300 Bruker NMR spectrometer equipped with an ASPECT 2000 computer on which mathematical manipulations were carried out. Chemical shifts are quoted downfield from the methyl resonance of sodium 2,2-dimethyl-1-silapentane 5-sulphonate but dioxane was used as an internal standard.

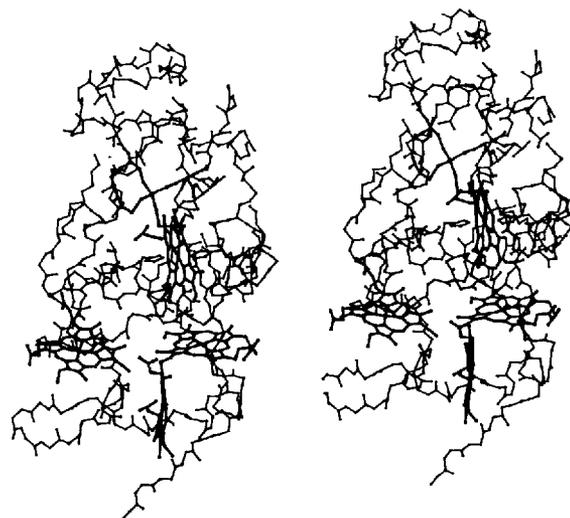


Fig. 1. Stereo view of the proposed complex between cytochrome c_3 and rubredoxin. The hemes of the cytochrome are shown along with the iron center of rubredoxin. The main-chain atoms of all residues in both structures are shown. In addition, all heavy atoms of the bridging ionic residues are shown

Table 1. Intramolecular bonds formed between cytochrome c_3 and rubredoxin

| Type of bond | Rubredoxin | Cytochrome c_3 | Distance |
|--------------|------------|------------------|----------|
| | | | nm |
| Salt bridge | Asp19 | Lys58 | 0.30 |
| | Glu17 | Lys57 | 0.30 |
| | Glu12 | Lys101 | 0.30 |
| | Glu50 | Lys95 | 0.30 |
| Hydrogen | Pro40 | Lys60 | 0.32 |
| | Val41 | Lys72 | 0.30 |
| | Thr7 | Lys94 | 0.30 |

RESULTS

Model building

As can be seen in Fig. 1, in the model proposed the iron atom of rubredoxin is in close (0.64 nm) proximity to heme 1 of cytochrome c_3 . Due to the large number of negatively charged residues on rubredoxin in the vicinity of the iron atom, this region has a large net negative charge. The intermolecular bonds formed in the complex shown in the figure are listed in Table 1. There are a total of seven intermolecular bonds formed: four salt linkages between lysine residues of cytochrome c_3 and acidic residues of rubredoxin and three hydrogen bonds between lysine residues of the cytochrome and main-chain carbonyl oxygen atoms of rubredoxin. All are within standard bond length limits.

NMR

We have been extensively using NMR techniques in order to analyze the highly resolved spectra obtained in two favorable spin states of tetraheme cytochrome c_3 : oxidized paramagnetic ($S = 1/2$) Fe(III) and reduced diamagnetic ($S = 0$) Fe(II) [13, 14, 43, 44]. The observed spectral enhanced resolution is a consequence of the low number of amino acids and the higher number of redox centers present per molecule.

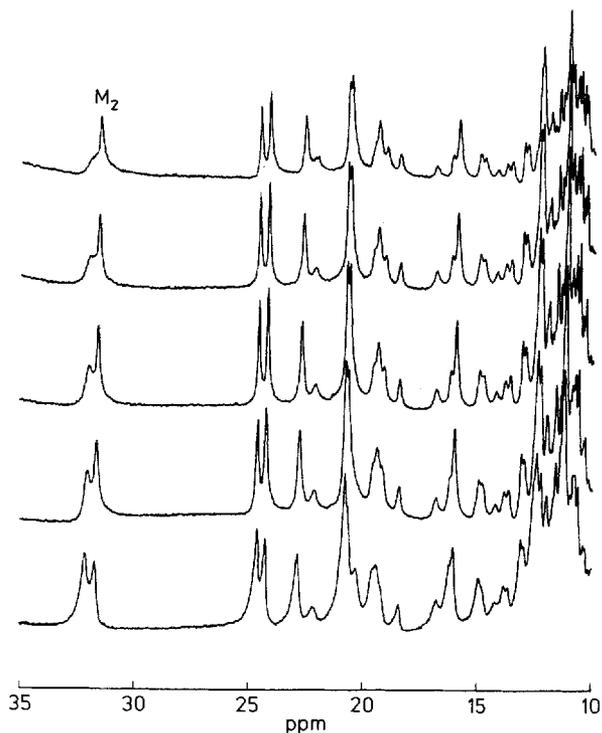


Fig. 2. NMR protein-protein titration. *D. vulgaris* (Hildenborough) cytochrome c_3 titrated with rubredoxin at 303 K, pH 8.10. The cytochrome c_3 concentration was 1.04 mM in the bottom spectrum. Titration was performed using a rubredoxin stock solution of 1.78 mM. Spectra (2000) scans were recorded at the following rubredoxin/cytochrome c_3 molar ratios (bottom to top): 0, 0.274, 0.478, 0.753 and 1.23

Being such a small protein, the polypeptide chain barely covers the four hemes and most of the amino acid residues are in the vicinity of the iron centers. The most striking features of the NMR spectra of multiheme cytochromes are the presence of resonances outside the range +11 ppm to -2 ppm for the ferric form and their absence in the ferrous form. These resonances arise from proton groups in the heme vicinity and thus experience large shifts due to the paramagnetic, ferric ions.

In the low-field region of the NMR spectrum, several proton resonances have been assigned to heme methyl resonances based on chemical shift, line width, intensity, and redox behavior, and have been used for probing the operating electron-transfer mechanisms [43, 44]. The NMR spectrum of *D. vulgaris* (Hildenborough) cytochrome c_3 has been previously discussed. Shown in Fig. 2 are detailed regions of the low-field part of the ^1H -NMR spectrum of this multiheme cytochrome and the effect of increasing amounts of rubredoxin isolated from the same organism. Selective modifications are immediately observed upon titrating cytochrome c_3 with the interacting proteins (Figs 3 and 4). The plots shown in Fig. 3A indicate that there are selective alterations of the chemical shift smaller than 0.0326 ppm which correspond to the resolution achieved between two data accumulation points. Also striking is the extensive broadening of heme methyl resonance 1 (Fig. 2 and Fig. 3B) upon increasing the rubredoxin/cytochrome c_3 ratio. This remarkable effect is the result of the perturbation of relaxation on methyl 1 introduced by the magnetic moment of high-spin rubredoxin Fe(III) center ($S = 5/2$). This perturbation implies that a

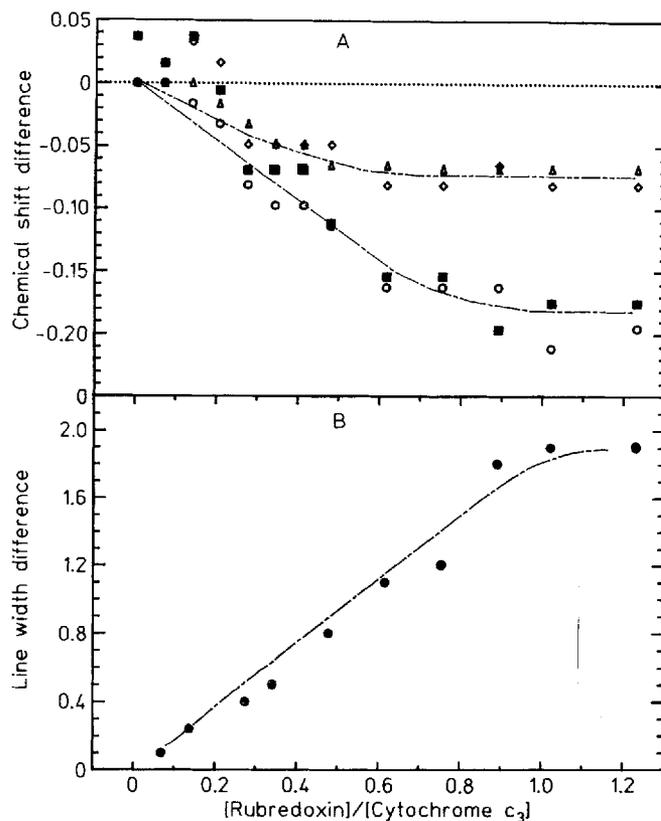


Fig. 3. Effect of rubredoxin binding on the NMR spectral parameters of cytochrome c_3 . NMR data was obtained under the same experimental conditions as indicated in the caption to Fig. 2. (A) Differences in chemical shifts of selected heme methyl resonances. (○) Methyl resonance 5; (■) methyl resonance 1; (△) methyl resonance 8; (◇) methyl resonance 2. (B) Variation of the half-width of heme methyl resonance 1

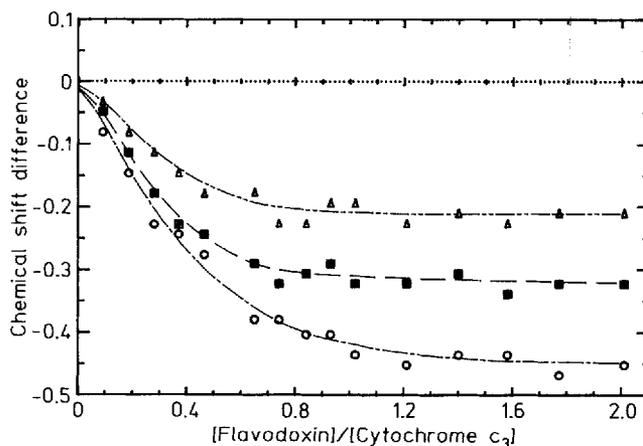


Fig. 4. Effect of flavodoxin binding on the chemical shifts of heme methyl resonances of cytochrome c_3 . NMR experiments were performed as described in the text and the legend of Fig. 2. (○) Methyl resonance 1; (■) methyl resonance 5; (△) methyl resonance 8

heme methyl group is in close proximity to the rubredoxin iron center. Note that, due to the increase of the tumbling time of cytochrome c_3 in solution upon complex formation, there is also a general trend for increasing line width in the overall spectral features, but in an unspecified mode.

Table 2. Chemical shifts of heme methyl resonances of cytochrome c_3 due to interaction with rubredoxin of flavodoxin

All values are maximal chemical shift differences detected upon complex formation. Values indicated in parenthesis were judged to be within or at the limit of spectral resolution

| Interacting partner | Methyl resonance | δ ppm |
|---------------------|------------------|-----------------|
| Rubredoxin | 1 | -0.16 |
| | 5 | -0.19 |
| | 8 | (-0.07) |
| | 2 | (-0.07) |
| Flavodoxin | (4) | (-0.04) |
| | 1 | -0.45 |
| | 5 | -0.32 |
| | 8 | -0.25 |
| | (3) | (-0.05) |
| | (7) | (-0.08) |

The observed alterations in the chemical shift are due to the specific interaction between rubredoxin and cytochrome c_3 . An analogous perturbation is seen for the interaction of flavodoxin with cytochrome c_3 (Fig. 4). In this case, three of the four resonances are similarly affected, but with even larger chemical shifts. These spectral perturbation cannot be due to pH shifts and indicate that for both proteins the same binding site is involved.

It can also be concluded that cytochrome c_3 is in fast exchange between the complexed form and the free unbound form on the NMR time scale. The observed trend in chemical shift alterations (Figs 3 and 4) indicates a 1:1 complex formation. The estimated maximum value for chemical shift differences between bound and unbound situations is indicated in Table 2. The analysis of the experimental data as a function of mole fractions of the two interacting partners enables the calculation of the binding constant: rubredoxin/cytochrome c_3 ($\approx 10^4 \text{ M}^{-1}$) and flavodoxin/cytochrome c_3 ($\approx 10^3 \text{ M}^{-1}$) at 303 K.

DISCUSSION

Aside from the NMR data, the strongest part of the argument for the previously proposed flavodoxin-cytochrome c_3 complex [11] is complimentary electrostatic interaction. With rubredoxin, on the other hand, the electrostatic potential arguments are less stringent. Since previous NMR studies with proteins from *Desulfovibrio gigas* [20] indicate that flavodoxin and rubredoxin do not bind to the same site in that species, initial efforts at modeling the rubredoxin-cytochrome c_3 complex focused on the other hemes. A possible docking site was revealed at heme 4 with six specific ionic and hydrogen bonds (unpublished results). With this heme 4 docking, the main heme interaction with rubredoxin is a salt bridge from the heme propionate moiety to lysine 46 of rubredoxin (a highly conserved residue in the known sequences). This model was characterized by a minimum heme–non-heme iron distance of 1.5 nm, but with the intervening space occupied mostly by aromatic residues. Thus, two possible docking sites were identified (heme 1 and heme 4). In both cases, a large degree of complimentary interaction was found between lysine residues on the cytochrome and acidic residues on the rubredoxin. However, the model with the heme 1 site involves much more

direct heme–non-heme iron interaction (Fig. 1). The NMR studies reported here were supported by the modeling efforts and show conclusively that, unlike the case with the *D. gigas* proteins, both rubredoxin and flavodoxin interact with the same heme group in *D. vulgaris* (Hildenborough). In addition, the significant broadening of heme methyl resonance 1 in the presence of rubredoxin (Fig. 3B) implies close proximity between the heme and non-heme iron centers. The iron–heme distance of 0.64 nm in this model satisfies this requirement and can account for the broadening observed upon complex formation. Thus, the model for interaction of rubredoxin at the heme 1 site is supported by both spectroscopic data and electrostatic calculations and, correspondingly, it gives a structural assignment to the spectroscopic features associated with this interaction.

Previous NMR experiments [43] on the reoxidation pattern of *D. vulgaris* (Hildenborough) cytochrome c_3 assigned heme methyl resonance 1 to a methyl group of one of the hemes with the least negative redox potential. This is the same resonance so strongly effected by binding of rubredoxin and flavodoxin (Table 2).

Stellwagen [45] has found that a correlation apparently exists between redox potential and heme exposure for cytochromes c : as the exposure decreases, the redox potential becomes more positive. While this is not the only factor responsible for heme potential as recently discussed by Moore et al. [46], in this case all four hemes have the same ligating amino acids (histidines) and the heme crevices have similar environments [7]. Thus, surface exposure may be an important factor and, according to these arguments, the least negative heme should also be the least exposed. Indeed, surface area calculations show that heme 1 is the least exposed.

The NMR data indicates that a 1:1 complex of rubredoxin and cytochrome c_3 are formed *in vitro* and that rubredoxin interacts with a specific heme of cytochrome c_3 . The specificity may be due to steric factors, electrostatic forces or redox properties of the heme. However, since at least one other docking site is supported by a similar set of specific, complimentary interactions (as discussed above), the specificity of the heme 1 site must lie in more general features of the interaction. One clue can be found in the electrostatic analysis of the region surrounding each heme as calculated in the investigation of the flavodoxin-cytochrome c_3 interaction (Fig. 1) [11]. The electrostatic potential field of heme 1 differs drastically from that of the others [11]. It is almost entirely positive and therefore would strongly attract a negatively charged region of a redox partner. The regions surrounding the other hemes have both negative and positive charges interspersed, offering less attraction and more repulsion to a uniformly negatively charged redox site on a docking protein. Flavodoxin is found to have a strikingly asymmetric surface charge, with a large negatively charged region surrounding its prosthetic group and the other regions of the surface possessing positive charge [11]. This type of asymmetrical charge distribution is also seen for rubredoxin, but to a lesser extent. Thus, predocking orientation forces should tend to favor the interactions proposed. This complex has a high degree of complementary interaction, as all of the lysine residues surrounding heme 1 of cytochrome c_3 are involved in either salt bridges or hydrogen bonds to rubredoxin.

The binding constant found for the rubredoxin/cytochrome c_3 complex compares with those found for other complexes, both physiological and non-physiological (Table 3). The proposed complex has seven intermolecular bonds while the flavodoxin complex has five. In a crude sense,

Table 3. Comparison of association constants from other studies with those calculated from this investigation

| Redox pair | Ionic strength | Binding constant | Reference |
|---|----------------|------------------|-----------|
| | M | | |
| Tuna cytochrome <i>c</i> /yeast cytochrome <i>c</i> peroxidase | 0.01–0.20 | 10^6 – 10^3 | [50] |
| <i>Azotobacter vinelandii</i> flavodoxin/horse cytochrome <i>c</i> | 0.088–0.17 | 10^4 – 10^3 | [51] |
| Beef cytochrome <i>b</i> ₅ /horse cytochrome <i>c</i> | 0.01–0.001 | 10^4 – 10^6 | [52] |
| Horse cytochrome <i>c</i> / <i>Clostridium pasteurianum</i> flavodoxin | 0.076 | 10^5 | [29] |
| <i>D. desulfuricans</i> Norway cytochrome <i>c</i> ₃ /ferredoxin | 0.01 | 10^6 | [16, 44] |
| <i>D. vulgaris</i> flavodoxin/cytochrome <i>c</i> ₃ | <0.005 | 10^3 | this work |
| <i>D. vulgaris</i> rubredoxin/cytochrome <i>c</i> ₃ | <0.005 | 10^4 | this work |

this correlates with the higher experimental binding constant of this complex. Thus it may be that the types of interaction proposed here, and previously for flavodoxin [11], are characteristic of the physiological interactions of cytochrome *c*₃, allowing the use of these hypothetical complexes as models for *in vivo* complexes between non-heme iron centers and flavoproteins with hemoproteins.

Comparison with other models

Several other complexes of electron-transport protein in *Desulfovibrio* have been studied. One of these, discussed above, is the flavodoxin-cytochrome *c*₃ complex of *D. vulgaris* (Hildenborough) [11]. Another interaction studied for which a model has been proposed is the ferredoxin-cytochrome *c*₃ complex from *D. desulfuricans* Norway [19]. For that study, the X-ray structure of *Pseudomonas aerogenes* ferredoxin was transformed into the *D. desulfuricans* structure, a difficult task since the two proteins show only 26% sequence similarity and the *P. aerogenes* protein contains two 4Fe-4S clusters as opposed to one in the *D. desulfuricans* protein. Features of the proposed complex include the proximity of the 4Fe-4S cluster of ferredoxin to a heme of the cytochrome and the formation of a 1:1 complex stabilized by interactions between acidic residues on the surface of ferredoxin and lysine residues surrounding the heme crevice of cytochrome *c*₃. In that complex, ferredoxin is also docked to the heme with the least negative redox potential [19]; however, ferredoxin perturbs the ¹H-NMR signal of the two highest potential hemes [47] indicating that both are involved in the interaction in some manner. An association constant of 10^6 M⁻¹ has been calculated at ionic strength 10 mM [47].

As mentioned, an interesting interaction for which only preliminary NMR data has been obtained [20] is the complex formed between *D. gigas* cytochrome *c*₃ and flavodoxin or rubredoxin. In contrast to the model presented here and our companion paper [11], the *D. gigas* data suggests that its rubredoxin and flavodoxin interact with different hemes: rubredoxin with the heme with the least negative redox potential and flavodoxin with the heme with the next highest redox potential (J. J. G. Moura, unpublished results). The structure of *D. gigas* rubredoxin has only recently been solved [5] but the structure of its cytochrome *c*₃ has not yet been determined. We are currently attempting to predict the structure of *D. gigas* cytochrome *c*₃ and flavodoxin using extensions of a method developed in this laboratory [48]. Such studies should help elucidate the factors responsible for directing the redox patterns of cytochrome *c*₃ to the appropriate heme.

One point which should be addressed is the acceptability of correlating NMR data from the Hildenborough strain of

D. vulgaris with the structure of cytochrome *c*₃ from the Miyazaki strain. Comparison of the sequences of the proteins from the two strains reveals a high degree of conservation of amino acids, with only 14 of 104 residues differing, none of which are involved in the complex. In addition, NMR studies [48] (K. Niki, personal communication) show that while considerable differences in the chemical shifts in the low-field region are seen while comparing the spectra of most of the cytochromes *c*₃, the spectra from the Hildenborough and Miyazaki strains of *D. vulgaris* are essentially identical. Furthermore, on the basis of a structural prediction method which allows the change of one protein into another by substitution of amino acid residues [49], it is expected that no significant structural differences relevant to the proposed complexes should exist between the two molecules.

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