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# Cytochrome $c_3$-ferredoxin I covalent complex: evidence for an intramolecular electron exchange in cytochrome $c_3$

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***Desulfovibrio desulfuricans*** **Norway tetraheme cytochrome $c_3$ and (4Fe-4S) cluster containing ferredoxin I are physiological electron-transfer partners. To obtain structural informations on the complex formation, a covalent cytochrome $c_3$-ferredoxin I complex was used. EPR investigations suggest that the covalent cytochrome $c_3$-ferredoxin I complex is a stable analogue of the native one and bring experimental support to an intramolecular electron exchange in cytochrome $c_3$. The reduction of cross-linked ferredoxin I under a hydrogen atmosphere in the presence of hydrogenase necessitates an intramolecular electron exchange between the hemes of cytochrome $c_3$. A spectrophotometric redox titration indicates that the redox potentials of cytochrome $c_3$ are weakly affected by the complex formation.**

Oxido-reduction reactions between two metalloproteins necessitate the formation of an intermediate complex in which the redox centers of the two proteins are optimally oriented to achieve physiological electron transfer [1]. The specificity of the recognition is provided by attractive electrostatic interactions involving the $\epsilon$-amino groups of basic amino-acid residues and the carboxyl groups of the acidic amino-acid residues of the partners. Water-soluble carbodiimide has proved to be useful for the covalent cross-linking of charges-paired functional protein–protein complexes. To date, cross-linked complexes have been obtained involving monohemic cytochrome *c* [2–8] or ferredoxin [9–11] and their oxidoreductases. They have been shown to share many properties with the physiological noncovalent complexes: same stoichiometry, isoelectric point, physio-

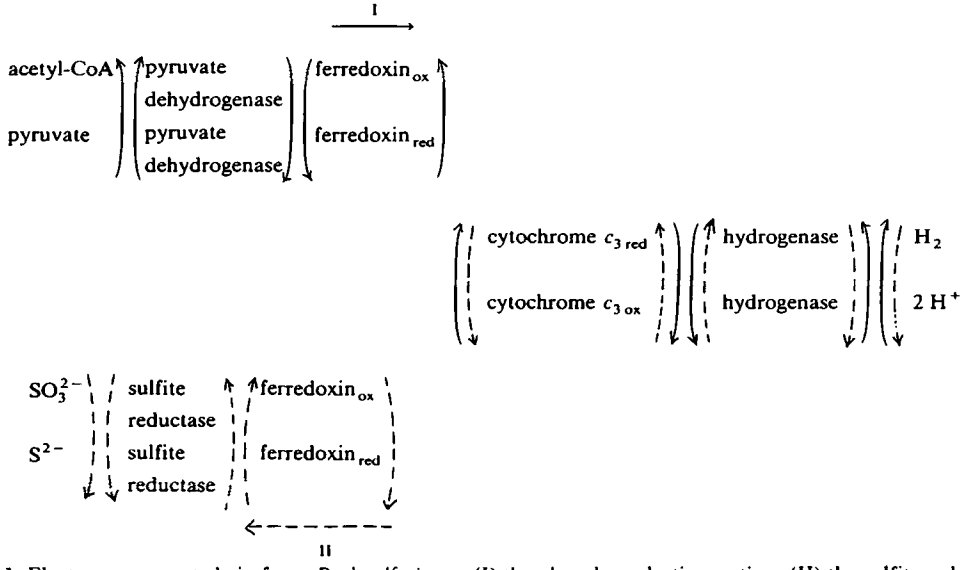


Fig. 1. Electron-transport chain from *D. desulfuricans*: (I) the phosphoroclastic reaction; (II) the sulfite reduction.

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logical activity and similar spectral perturbations. Covalent cross-linking is a convenient technique to recognize the structural features required for the optimization of the electron transfer. In an attempt to elucidate the role of the different hemes in the tetrahemic cytochrome $c_3$, we have performed cross-linking experiments of this cytochrome with its redox partner, the ferredoxin isolated from *Desulfovibrio desulfuricans* Norway [12]. Cytochrome $c_3$ and ferredoxin act as obligate partners in the phosphoroclastic reaction and the sulfite reduction; the other partner of cytochrome $c_3$ is hydrogenase (Fig. 1) [13,14].

Both primary and tertiary structures of cytochrome $c_3$ ($M_r = 13\,000$) have been determined [15,16]. The four hemes, localized in non equivalent protein environments [17,18], exhibit different and low redox potentials [18–20]. Ferredoxin I possess one (4Fe-4S) cluster of redox potential −374 mV [21]. Primary structure and preliminary crystallographic data have been reported [22,23]. Rapid kinetic experiments have shown a bimolecular complex formation followed by a bidirectional electron exchange between cytochrome $c_3$ and ferredoxin [24]. The complex formation has been described using the oxidized proteins by $^1$H-NMR [25] and microcalorimetric measurements [26], with a stoichiometry 1 : 1 and an association constant $K_a = 1.3 \cdot 10^6\ M^{-1}$ (Tris-HCl, $10^{-2}$ M; pH 7.7, $T = 283$ K). The analysis of ionic strength dependence exhibited an important electrostatic effect on the association process [26]. The construction of the cytochrome $c_3$-ferredoxin complex model with computer-aided graphics has shown a large number of ion pairings between the two components [27]. The structural data [25,27] and chemical modification experiments [28] brought arguments for the identification of the highest redox potential heme, which was demonstrated to be the interacting heme in the complex, as heme 4 (sequence numbering). In view of an utilization of the cytochrome $c_3$-ferredoxin I covalent complex for obtaining functional and structural informations, a further characterization was attempted by EPR through a physiological reduction of the complex in presence of hydrogenase.

*Desulfovibrio desulfuricans* Norway cytochrome $c_3$, ferredoxin I and hydrogenase were purified as previously described [29,30]. The 1 : 1 cross linked complex between cytochrome $c_3$ and ferredoxin I was obtained as previously reported [12]. Incubation of cytochrome $c_3$ and ferredoxin I together with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluene sulfonate allows the formation of two components of $M_r = 38\,500$ and $M_r = 24\,000$, analyzed through electrophoresis on polyacrylamide gel in presence of SDS. The 1 : 1 complex ($M_r = 24\,000$) is further purified on an ultrogel ACA 54 column in Tris-HCl (0.05 M)-NaCl (0.5 M) buffer (pH 7.6). The optical spectrum of the cytochrome $c_3$ in the complex was identical to that of the native one. The concentration of the EPR sample was 0.5 mM. The final concentration of the sample used in the spectrophotometric titration was 15 μM.

A noncovalent complex was obtained from an equimolar mixture of oxidized cytochrome $c_3$ and ferredoxin I. Using an association constant of $10^6\ M^{-1}$ [26] it can be calculated that about 95% of the molecules are complexed in the EPR sample (protein concentration, 0.5 mM), and about 80% in the sample used in the spectrophotometric titration (protein concentration, 15 μM).

A sample of native cytochrome $c_3$ was used as a reference. Its concentration was 0.5 mM for EPR and 10 μM for the spectrophotometric titration. All the samples were buffered in 10 mM Tris-HCl (pH 8.1). The reduction of the EPR samples was obtained by addition of hydrogenase (5 μM) after pressurization with argon for 20 min and then with hydrogen for 10 min.

The EPR spectra were recorded at 16 K on a Varian E 112 X band spectrometer equiped with an Air-Products Helitran Helium gas flow system. The spectrophotometric titration was performed at 26 °C, and was followed by absorbance measurements at 550 nm with a Beckman DU40 spectrophotometer. The mediators were identical to those used in Ref. 20.

*EPR Spectra.* In the oxidized state, the EPR spectra of the covalent (Fig. 2) and noncovalent (data not shown) complexes are very similar to that given by cytochrome $c_3$ [18]. Neither the complexation nor the cross-linking process seems to affect the environment of the four hemes noticeably. Reduction by hydrogenase under $H_2$ atmosphere leads to the disappearance of the heme signals and the appearance of a typical signal of reduced (4Fe-4S) cluster, for both the covalent and the noncovalent complexes (Fig. 3). The signal given by the reduced (4Fe-4S) cluster in the covalent complex is slightly different in the high-field region from that given by the noncovalent complex (Fig. 3), which is identical

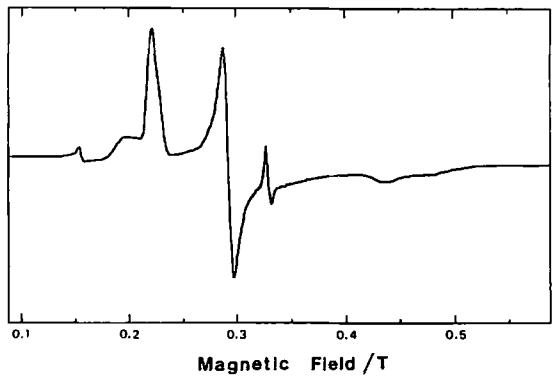


Fig. 2. EPR spectrum of the cytochrome $c_3$-ferredoxin I covalent complex in the oxidized state. Experimental conditions: $T = 16$ K; microwave power, 4 mW at 9300 MHz; field modulation, 10 G p.p. at 100 kHz.

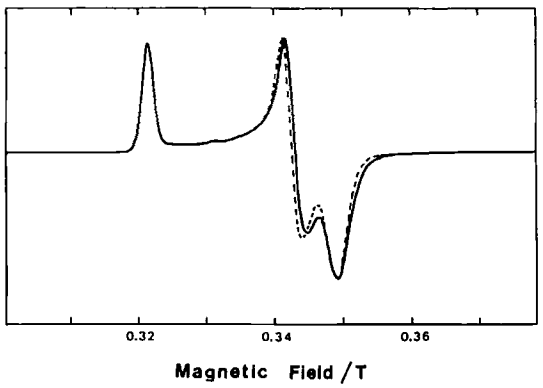


Fig. 3. EPR spectrum of the cytochrome $c_3$-ferredoxin I covalent (———) and noncovalent (- - - - - -) complexes in the reduced state. Same conditions as in Fig. 2.

to that of ferredoxin I [21]. By performing numerical double integrations on the signals given by the covalent complex, we found that the ratio of the number of $S = 1/2$ species present in the oxidized state and in the reduced state is equal to $3.8 \pm 0.4$. This indicates that in all the complexes the (4Fe-4S) cluster is present and has been reduced. As we have observed that ferredoxin I cannot be reduced by hydrogenase [14], this result implies the participation of the hemes in the reduction of the cluster.

Cross-linking reaction does not apparently induce conformational changes of the redox centers. Orientation and distances between heme and iron sulfur cluster in the covalent complex are favorable to the electron transfer. Then, the covalent cytochrome $c_3$-ferredoxin I complex appears to be a stable analogue of the native one.

*Spectrophotometric titration.* The results of spectrophotometric redox titration followed by absorbance measurements at 550 nm are represented in Fig. 4 for the free cytochrome $c_3$, the noncovalent and the covalent complexes.

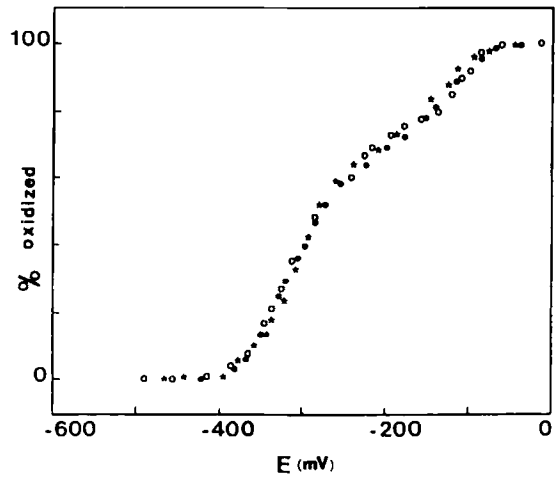


Fig. 4. Spectrophotometric redox titration of cytochrome $c_3$ (○), cytochrome $c_3$-ferredoxin I covalent complex (*), cytochrome $c_3$-ferredoxin I noncovalent complex (●).

We have already reported the results of a titration of the free cytochrome $c_3$, which were in agreement with those obtained by EPR measurements [20]. The data represented in Fig. 4 for the free cytochrome $c_3$ are very similar to those published previously, although a slight positive shift of about 20 mV is apparent in the potential range corresponding to the reduction of the highest potential heme. This effect could be related to the differences between the temperatures (21° C and 26° C) in the two experiments, or to an insufficient equilibration between the solution and the electrode in this potential range. Although this effect deserves further study, the three sets of data represented in Fig. 4 have all been obtained under identical conditions and can thus be confidently compared. It appears that the redox properties of the hemes are not appreciably affected by the complexation or the cross-linking processes. Complexation with ferredoxin might be expected to induce an environmental change of the interacting heme group and therefore a modification of its redox potential value. Our result suggests that environmental changes, including solvent accessibility, charge neutralization at the interacting site and structural modification, compensate one another.

Moreover, it should be recalled that redox titrations based on absorbance measurements are only sensitive to the values of the macroscopic redox potentials. The results must then be considered as preliminary, and should be confirmed by a titration followed by a spectroscopic technique sensitive to the microscopic potentials like NMR [31] or EPR [20].

In the case of multiredox center proteins, two mechanisms of electron transfer have to be considered [32]:
- an intermolecular electron exchange between the redox centers of two molecules;
- an intramolecular electron exchange within the redox centers of the same molecule.

Because it contains four heme groups per molecule, cytochrome $c_3$ is a model for understanding such electron exchanges. Resonance Raman studies of hydrogenase-catalyzed reduction of *D. vulgaris* Miyazaki cytochrome $c_3$ by hydrogen have recently suggested that the electron exchange, either intramolecular within the four hemes or intermolecular between different cytochrome $c_3$ molecules, is much slower than $10^{12}$ $s^{-1}$. NMR data have suggested that the intramolecular electron exchange rate is faster than $10^9$ $s^{-1}$ and the intermolecular one slower than $10^4$ $M^{-1} \cdot s^{-1}$ [33]. Analysis of the NMR reoxidation pattern of both *D. vulgaris* Hildenborough and *D. gigas* cytochromes $c_3$ have suggested the existence of slow intermolecular and fast intramolecular electron exchanges (larger than $10^5$ $s^{-1}$) on the NMR time scale [31,34]. On the contrary, evidence was found for fast intermolecular electron exchange within the four hemes of *D. desulfuricans* Norway cytochrome $c_3$ [17]. Rapid intramolecular elec-

tron exchange, which was proposed to interpret the results of kinetic investigations on *D. desulfuricans* Norway cytochrome $c_3$-ferredoxin I complex [24], was not observable through $^1$H-NMR studies [17], probably due to the large difference between the redox potentials of the individual hemes.

The present study brings experimental support to this intramolecular electron exchange. The most obvious result is the ferredoxin (4Fe-4S) cluster reduction in presence of hydrogenase in the cytochrome $c_3$-ferredoxin I covalent complex. Cytochrome $c_3$ is an obligate intermediate between ferredoxin and hydrogenase in the electron-transport chains. As indicated by model building of the complex with computer-aided graphics [27], the cytochrome $c_3$ heme 4 is buried, facing the ferredoxin iron-sulfur cluster. In the covalent complex, hydrogenase has to give its electron(s) to the cytochrome molecule through one of the three other hemes. Thus, two hypotheses can be formulated:

- the high-affinity interacting site for hydrogenase on cytochrome $c_3$ is different from the ferredoxin one;
- the high-affinity interacting site for hydrogenase and ferredoxin is the same but there are one or several other sites of low affinity that could allow the electron exchange.

However, in the two cases, reduction of cross-linked ferredoxin I in the covalent complex necessitates an intramolecular electron transfer between at least two cytochrome $c_3$ hemes, one being the reactive heme group facing the ferredoxin (4Fe-4S) cluster and the other one being the heme which receives electron(s) from hydrogenase.

Crystallographic results have suggested electron-transfer pathways within the redox core [35]. Direct electron transfer could take place through electron orbital overlaps with a possible involvement of aromatic residues (Phe 34, Phe 88). The importance of the $\alpha$ helix dipole in the electron exchange between heme 3 and heme 4 has also been proposed [36]. More information about the intramolecular electron transfer pathway should be obtainable by using chemical modifications or site-directed mutagenesis.

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