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Identification of the site of interaction between cytochrome c_3 and ferredoxin using peptide mapping of the cross-linked complex

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Structural studies carried out on a cross-linked complex between cytochrome c_3 and ferredoxin I, both isolated from *Desulfovibrio desulfuricans* Norway, allowed the identification of the site of interaction between the two redox proteins. *Staphylococcus aureus* proteinase and chymotrypsin digestions led to characterization of peptides containing both cytochrome c_3 and ferredoxin sequences. The cytochrome c_3 sequences involved in the three isolated cross-linked peptides contained several lysine residues localized around the heme 4 crevice. This analysis stressed the peculiar role of lysines 100, 101, 103, 104 and 113, which could be considered as major cross-link sites, as opposed to the lysines 75, 79 and 82, which could be considered as minor cross-link sites. One cross-linked peptide, containing two ferredoxin sequences joined to one cytochrome c_3 sequence, had been isolated, suggesting the possibility of more than one cross-link per covalent complex. All these results led to the identification of heme 4 of cytochrome c_3 as the site of interaction for the ferredoxin I. This study confirms the proposal that could be deduced from the hypothetical structure of the complex built by computer graphics modelling (Cambillau, C., Frey, M., Mosse, J., Guerlesquin, F. and Bruschi, M. (1988) Proteins: struct., funct. genet. 4, 63–70).

Introduction

Electron transfer reactions are fundamental to important biochemical pathways such as respiration or photosynthesis. The mechanism by which electrons are transferred between electron carrier proteins has been reported to necessitate the formation of an intermediate complex in which the redox centers of the two redox proteins are optimally oriented to achieve physiological electron transfer [1]. The three-dimensional structures of the two proteins are involved in the control of the electron transfer rate from one metalloprotein to another. Unfortunatly, no three-dimensional structure of electron transfer complexes has yet been determined by X-ray analysis, except for the photosynthetic reaction center [2,3].

Some of the more thoroughly studied electron transfer reactions are those involving cytochrome *c*. Indirect methods, such as chemical modifications [4–8], biophysical experiments [9–11] chemical cross-linking [12–17] and computer graphics modelling [1,18,19], have been used to elucidate the intermolecular interface of these electron-transfer complexes.

We have focused our study on the cytochrome c_3-ferredoxin I complex, isolated from the sulfate-reducing bacterium *Desulfovibrio desulfuricans*, Norway.

Tetraheme cytochrome c_3 is a cofactor for the *Desulfovibrio* hydrogenase as it is required for the reduction of ferredoxin, flavodoxin or rubredoxin by hydrogenase plus hydrogen. 'In vitro' specific interactions have been demonstrated for these proteins [20,21]. *Desulfovibrio* ferredoxins are described as low-potential redox proteins active in stimulating hydrogen evolution from pyruvate and hydrogen utilization with sulfite [22]. Two homologous ferredoxins have been described in *D. desulfuricans* Norway, one of the four-iron type (ferredoxin I) and the other of the eight-iron type (ferredoxin II), that are equally active in hydrogen uptake and evolution [23]. NMR and EPR data suggest a specific interaction between cytochrome c_3 and ferredoxin I [24,25] and a similar observation has been made concerning cytochrome c_3 and ferredoxin II purified from *Desulfovibrio gigas* [21]. Recent results on cellular locali-

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zation [26,27] pointed out that such a reaction between cytochrome c_3, which is located in the periplasm, and ferredoxin, found in the cytoplasm, is not physiologically relevant. However, the real physiological reaction could take place between a transmembrane cytochrome which has retained some analogies with the tetraheme cytochrome c_3, [28] or that ferredoxin does not necessitate a signal peptide for translocation to the periplasm. In an attempt to investigate the structure–function relationships in multiheme cytochrome c_3, we have developed the study of the electron transfer mechanism between *D. desulfuricans* Norway cytochrome c_3 and ferredoxin I as a model of the study of electron transfer reactions involving hemes and non-heme iron clusters.

Both primary and tertiary structures of cytochrome c_3 (M_r 13000) have been determined [29,30]. The four hemes, localized in distinct protein environments [31,32,33], exhibit different and low redox potentials, ranging from −150 mV to −400 mV [34,35]. Ferredoxin I possess one [4Fe-4S] cluster per subunit (M_r 6000) [36] of redox potential −374 mV [37]. Although a two-dimensional NMR study is in progress [38], the complete resolution of the structure is not yet solved.

Rapid kinetics experiments have demonstrated the formation of an intermediate complex followed by a bidirectional electron transfer between heme and [4Fe-4S] cluster [39]. The complex formation has been described using the oxidized proteins by ^{1}H-NMR [24] and microcalorimetric measurements [40], and a corresponding stoichiometry 1 : 1 with an association constant $K_a = 1.3 \cdot 10^6$ M^{-1} (10 mM Tris-HCl (283 K; pH 7.7)) has been determined. Although the microcalorimetric parameters were consistent with a hydrophobic process being involved in the complex formation, the ionic strength dependence demonstrated a significant electrostatic effect on the association [40]. It was also found that presence of ferredoxin I induced specific cytochrome c_3 ^{1}H-NMR spectrum changes on the two highest redox potential heme ring methyl lines [24].

A hypothetical structure of the cytochrome c_3-ferredoxin I complex was generated, using interactive computer graphics methods, based on the X-ray structure of the cytochrome c_3 and a model of ferredoxin I derived from *Peptococcus aerogenes* ferredoxin [41]. Among the four possibilities, in which the [4Fe-4S] cluster faces each heme successively, the most favorable one, in terms of charge interactions and surface contact topology complementarity, involved heme 4 (sequential numbering). In this model, the closest distance between the respective prosthetic groups was 11.8 Å and five ion pairs between acidic groups of ferredoxin and basic groups of cytochrome c_3 were formed [41].

Two hypothetical complexes involving cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki and both flavodoxin and rubredoxin from *D. vulgaris* Hildenborough were generated and suggested that the same heme, namely heme 4 (sequentially numbering), is the flavodoxin or the rubredoxin interacting site [42,43]. This cytochrome is homologous to the *D. desulfuricans* Norway one and these studies suggest that the same heme (heme 4) is the reactive heme for ferredoxin, rubredoxin or flavodoxin.

The correlation between the cytochrome c_3-ferredoxin I hypothetical complex and the biophysical data, together with the chemical modifications experiments on cytochrome c_3 [44,45], leads us to propose the identification of the highest redox potential heme (−165 mV) as heme 4 for the ferredoxin interacting heme.

In order to obtain more structural information on the ferredoxin interacting site on cytochrome c_3, a cross-linked cytochrome c_3-ferredoxin I complex was synthesized using a carbodiimide as bifunctional reagent [46]. This cross-linking agent was chosen because it is expected to catalyse the formation of amide bonds between surface carboxylate and amino groups of ferredoxin I and cytochrome c_3, respectively, these types of group being involved in salt bridges as suggested by the microcalorimetric measurements [40] and the computer graphics modelling [41]. The covalent complex may be considered as a valid model of the non-covalent electron transfer complex on the following basis:

(i) properties of the native complex are conserved in the cross-linked one (spectral shapes, ionic strength dependence) [46];

(ii) electron transfer between heme and [4Fe-4S] cluster occurs in the cross-linked complex as well as in the native one [47].

Although several structural studies on protein–protein interactions using chemical cross-linking have been performed, only in the case of the cytochrome *c*-cytochrome *c* peroxidase [12] and ferredoxin-ferredoxin $NADP^+$ reductase [48] complexes has identification of the cross-linked peptides allowed the interacting domain to be mapped. In this paper, we report the structural studies carried out on the covalent complex with the aim of identifying the peptide regions of the two proteins that are interfaced in the complex. The results are correlated with the hypothetical structure of the cytochrome c_3-ferredoxin I complex.

Materials and Methods

Cytochrome c_3 and ferredoxin I from *Desulfovibrio desulfuricans* Norway were purified as previously described [49]. The cross-linked complex between the two proteins was obtained as previously reported [46], except for the last step in which the reaction mixture is directly chromatographed on an Ultrogel ACA54 column without addition of acetate for the reaction quenching.

Purification of cross-linked peptides

Apoprotein was obtained by incubating, with agitation, the complex (2 mg/ml) with 8 M urea in 0.1 M HCl and an $HgCl_2$ 5-fold excess (w/w) overnight at 37° C. Then the reaction mixture was chromatographed on a Sephadex G25 column equilibrated and eluted with 5% formic acid to separate complex depleted of prosthetic groups and reagents [50]. The *S*-carboxymethylated complex was prepared by dissolving the complex in 0.5 M Tris-HCl (pH 9.0)/8 M urea/20 mM EDTA, and treating it with iodoacetic acid according to Crestfield et al. [51].

The complex was digested with chymotrypsin in 0.2 M ammonium acetate solution (pH 8.5). A freshly prepared aqueous solution of the enzyme (1 mg/ml) was added to give an enzyme-to-substrate ratio of 1 : 50 (w/w). After incubation 3 h at 37° C, the digestion was stopped by freezing.

Digestion by *Staphylococcus aureus* proteinase was carried out in 0.2 M ammonium acetate (pH 8.5) by addition of enzyme solution (1 mg/ml) to give an enzyme-to-substrate ratio of 1 : 30 (w/w) for 90 min at 37° C. A second and then (90 min later) a third identical amount of the enzyme solution was added to the digestion mixture. Hydrolysis was stopped by freezing after a total time of 5 h.

The digests were taken to dryness in a vacuum centrifuge and then dissolved in the HPLC starting buffer (0.06% CF_3COOH). Peptides were separated by reverse-phase HPLC on a column Vydac C18 which was eluted with a linear gradient of 0–40% acetonitrile in 0.06% CF_3COOH (room temperature, 1 ml/min flow rate). The elution profile was monitored at 226 nm. Peptides to be submitted to sequencing and amino-acid composition analysis were collected from duplicate or triplicate maps, taken to dryness with a vacuum centrifuge and frozen at −20° C.

Amino-acid and sequence analysis of the peptides obtained from S. aureus proteinase and chymotrypsin digestions

Peptide samples were hydrolyzed in 200 µl of 6 M HCl at 110° C for 18 h in sealed evacuated tubes and their amino-acid compositions were determined with a LKB Model 4150 Amino-acid analyzer. Microsequence analyses were obtained in an Applied Biosystems A470 gas-phase sequencer with 1–5 nmol amounts. The quantitative determination of phenylthiohydantoin derivatives was compared to known amounts of the appropriate standards by HPLC (Waters) as described by Bonicel et al. [52].

Localization of the cross-linked peptides on the cytochrome c_3 structure

Three-dimensional coordinates of *D. desulfuricans* Norway cytochrome c_3 were obtained from the Brookhaven data bank. The structure was visualized on a computer system using the PCDRA program (Afshar, M., Nanard, M., Sallantin, J. and Haiech, J., unpublished data).

Results and Discussion

Among the large number of studies involving the use of a carbodiimide to provide stable covalently coupled 1 : 1 complexes of electron transfer proteins, few have attempted to identify the cross-linked peptides through degradative techniques. This analysis is particularly difficult by reason of:

(i) the micro-heterogeneity of the cross-linked complex molecules, in terms of number and site of cross-links;

(ii) the different efficiency of degradation of cross-linked complexes by specific proteinases;

(iii) the resolution of multiple cross-linked peptides even by reverse-phase HPLC, as few studies concerning the separation of branched peptides have been reported.

In order to identify the contact sequences between ferredoxin and cytochrome c_3, structural studies have been carried out on the cross-linked complex, obtained by using 1-cyclohexyl-3-(2-morphodinoethyl)carbodiimide metho-*p*-toluenesulfonate (CME-CDI) [46]. The covalently bound proteins were digested with various proteolytic enzymes (chymotrypsin (C) and *S. aureus* proteinase (S)) and the resulting peptides were separated by reverse-phase HPLC. Very good reproducibility of the covalent complex elution profile was observed in several runs. In this paper, only the *S. aureus* proteinase digestion is reported and fully described.

Most of the peptide peaks were characterized by amino-acid composition and N-terminal sequence and were identified by comparison with the published sequences of the two purified proteins [29,36]. From the *S. aureus* proteinase digestion, six peptides (S-1 to S-6) were identified as cytochrome c_3 sequence-containing peptides (Table I). Moreover, sequential analyses of peptides S-4, S-5 and S-6 resulted in a release of two amino acids/cycle for at least four cycles. Analysis of these N-terminal sequences suggested that these peptides contained both cytochrome c_3 and ferredoxin I sequences. After further characterization through amino acid composition, these probably cross-linked peptides were identified as sequences Ala-53–Glu-71, Phe-72–Glu-85 and Asn-86–Asn-118 from cytochrome c_3 joined respectively to sequences Lys-31–Glu-42, Cys-43–Glu-54 and Thr-1–Glu-7 from ferredoxin I (Table I).

It is noteworthy that this peptide mapping permits recovery of the whole cytochrome c_3 sequence. Three parts of the ferredoxin sequence are joined to cytochrome c_3 ones; the remaining sequences have been recovered as small peptides that are not cross-linked to any cytochrome sequence.

One likely cross-linked peptide (C-1) could be identified from the covalent complex chymotrypsic digestion.

TABLE I

Amino-acid composition of the peptides isolated after the Staphylococcal proteinase digestion of the S-carboxymethylated cross-linked complex

Determined by amino acid analyses or (in brackets) derived from the sequence.

Peptides	S-1	S-2	S-3	S-4	S-5	S-6
Residues covered:	C3 1–14	C3 15–43	C3 44–52	C3 53–71 FD 31–42	C3 72–85 FD 43–54	C3 86–118 FD 1–7
Asp	3.0 (3)	1.0 (1)	0 (0)	3.0 (3)	4.8 (5)	5.0 (5)
Thr	0.7 (1)	2.4 (3)	1.6 (1)	3.9 (4)	0 (0)	5.5 (6)
Ser	0 (0)	0 (0)	0 (0)	1.8 (2)	0 (0)	0 (0)
Glu	1.0 (1)	1.7 (2)	2.1 (2)	2.1 (2)	2.7 (3)	2 (2)
Pro	2.0 (2)	2.6 (3)	0 (0)	0.9 (1)	1.0 (1)	2.1 (2)
Gly	1.1 (1)	1.9 (2)	0 (0)	3.2 (3)	0 (0)	2.2 (2)
Ala	2.8 (3)	2.8 (3)	0 (0)	4.7 (5)	3.8 (5)	3.0 (3)
Cys	0 (0)	0 (0)	1.8 (2)	1.8 (2)	1.5 (2)	3.8 (4)
Val	1.0 (1)	1.8 (2)	1.0 (1)	2.0 (2)	2.0 (2)	1.0 (1)
Met	0 (0)	0.6 (1)	0 (0)	0.8 (1)	0 (0)	0 (0)
Ile	1.1 (1)	0 (0)	0 (0)	0 (0)	1.8 (2)	2.6 (3)
Leu	0 (0)	0.8 (1)	1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)
Tyr	0.6 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phe	0 (0)	0.8 (1)	0 (0)	0 (0)	0 (0)	1.0 (1)
His	0 (0)	1.8 (2)	1.8 (2)	1.1 (1)	0 (0)	3.6 (4)
Lys	0 (0)	3.6 (4)	0 (0)	2.8 (3)	3.8 (4)	5.7 (6)
Arg	0 (0)	0 (0)	0 (0)	0 (0)	1.0 (1)	0 (0)
Yield	6%	20%	55%	48%	74%	3%

Sequencing of this peptide resulted in a release of three amino acids/cycle for at least five cycles (data not shown). Both N-terminal sequences and amino-acid composition were consistent with the presence of two ferredoxin I sequences (Thr-1–Phe-23 and Ala-24–Lys-31), probably cross-linked with the cytochrome c_3 sequence Lys-100–Asn-118.

In order to check whether these peptides are effectively cytochrome c_3-sequences cross-linked to ferredoxin ones, the chromatographic behaviour of these sequences in reverse-phase HPLC with a C_{18} column was predicted according to Sasagawa et al. [53]. The results, in terms of sequence retention times, are reported in Table II. This analysis suggests that peptide C-1 is composed of cytochrome c_3 and ferredoxin sequences that have distinct retention times. Similar cases obtained for peptides S-6 and S-5. Then these peptides seem to be effectively both cross-linked cytochrome c_3 and ferredoxin I sequences.

On the other hand, such analysis of peptide S-4 shows that the corresponding sequences have close retention times on the C_{18} column. This peptide could therefore correspond to a non-cross-linked mixture of cytochrome c_3 and ferredoxin I sequences.

This structural analysis suggests that peptides S-6 and S-5, obtained from *S. aureus* proteinase digestion,

TABLE II

Prediction of the retention times of the cross-linked sequences on a C_{18} column according to Sasagawa et al. [53]

Peptide	Cross-link sequences	Retention time (min)	Available cross-link sites	Salt bridge in the hypothetical complex
S-4	Cyt. Ala-53–Glu-71	57.50	Lys-59,60	
	Fd. Lys-31–Glu-42	52.57	Asp-38	
S-5	Cyt. Phe-72–Glu-85	37.27	Lys-75,79,82	Arg-73–Asp-49
	Fd. Cys-43–Glu-54	56.99	Asp-46,49	
S-6	Cyt. Asn-86–Asn-118	62.52	Lys-97,100,101,103,104,113	Lys-101–Asp-5
	Fd. Thr-1–Glu-7	49.86	Asp-5	
C-1	Cyt. Lys-100–Asn-118	55.87	Lys-100,101,103,104,113	Lys-100–Glu-29
	Fd. Ala-24–Lys-31	47.24	Asp-5,27	
	Fd. Thr-1–Phe-23	65.57	Glu-7,13,17,21,29,30	Lys-101–Glu-7,Asp-5

and peptide C-1, obtained from chymotrypsic digestion, can be used as a mark of the cytochrome c_3-ferredoxin I complex intermolecular interface.

Analysis of the intermolecular interface

Sequence analysis of the cross-linked peptides shows that each contains several basic (Lys) and acidic (Asp, Glu) residue pairs that could be cross-link sites (Table II). It is to be noticed that, within the cross-linked peptides S-6, S-5 and C-1, among the acidic and basic residue pairs that could be involved in amide bonds between the two proteins, there is one pair, or two pairs in the case of C-1 peptide, expected to form a salt bridge in the complex according to the graphics computer modelling [41].

Peptide C-1 is composed of one cytochrome c_3 sequence cross-linked to two ferredoxin ones (Table II). We can thus assume that, within a covalent complex molecule, several cross-links could be induced by the carbodiimide, leading to more than two N-terminal sequences per cross-linked peptide after proteolytic digestion.

Cross-linked peptide sequences suggest that one of the lysine residues 100, 101, 103, 104 and 113 could be involved in a major cross-link, as two cross-linked peptides obtained by different enzymatic digestion have been identified and contains these lysine residues. On the other hand, the other lysine residues (Lys-75, 79, 82) could be considered as minor cross-linked sites. This suggestion could be correlated to the identification, in the *S. aureus* proteinase digestion map, of the peptide corresponding to the Phe-72–Glu-85 cytochrome c_3 sequence, in both the free form (5% yield) and cross-linked to the Thr-1–Glu-7 ferredoxin sequence. Then we have to assume that, within the cross-linked molecule population, one or more of the lysines 100, 101, 103, 104 and 113 are cross-linked to one ferredoxin acidic residue in all the molecules, and one or more of the lysines 75, 79 and 82 are cross-linked to one ferredoxin acidic residue in only some cross-linked molecules. This analysis underlined the micro-heterogeneity of the covalent complex molecule population, in terms of sites and number of cross-links, and could be correlated to the isoelectric pattern of the covalent complex, previously described [46]. This micro-heterogeneity has also been reported on the cytochrome *c*-cytochrome b_5 cross-linked complex [17].

The localization of the cytochrome c_3 sequence corresponding to the cross-linked peptides S-6, S-5 and C-1 in the cytochrome structure has been determined using the PCDRA program (see Materials and Methods) (Fig. 1). It appears that the various cross-linked peptides are in part localized near the heme 4 crevice. Every cross-linked peptide contains lysine residues localized around

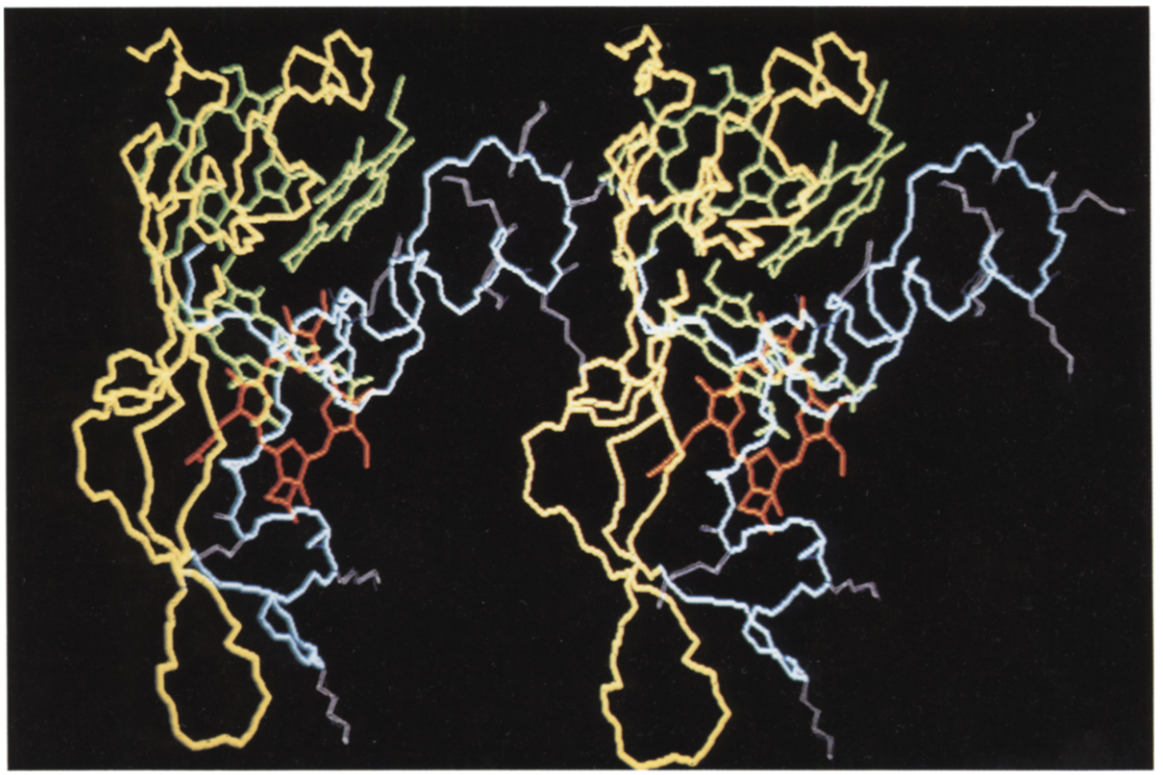

Fig. 1. Localization, in stereoview, of the three cross-linked peptides (S-5, S-6 and C-1) (in blue) in the three-dimensional structure of the cytochrome. Heme 4 is in red and the lysine residues, contained in these peptides, are in grey.

the heme 4 crevice; these lysines could be involved in amide bonds with acidic groups of ferredoxin I induced by the carbodiimide. We can therefore conclude that this part of the cytochrome is the ferredoxin-interacting domain and that heme 4 corresponds to the electron-transfer site for the [4Fe-4S] cluster of ferredoxin I.

This result confirms the proposal of heme 4 as the interacting site for ferredoxin I that could be deduced from the hypothetical structure of the complex built by computer graphics modelling [41]. However, it is to be noted that this modelling does not take into account the helical structure within the ferredoxin, recently determined by two-dimensional NMR [38]. In this case, we have to assume that this helical structure is running behind the molecule, so that the interacting site around the [4Fe-4S] cluster is not disturbed. These structural data are also in agreement with the three-dimensional structure of [4Fe-4S] cluster ferredoxin from *Bacillus thermoproteolyticus* elucidated by Fukuyama et al. [54] using X-ray crystallography. These authors have suggested that this helical structure, conserved in all mono-cluster ferredoxins, could be correlated to the second cluster deletion and has a possible role in maintaining the tertiary structure [54].

The correlation between this structural analysis and the biophysical experiments on the interaction suggests that one of the factors involved in the complex formation are electrostatic interactions between basic groups of cytochrome c_3 which surrounded heme 4 crevice and acidic groups of ferredoxin I which are localized around the [4Fe-4S] cluster.

In both computer graphics modelling and covalent cross-linking experiments, one of the important criteria in the complex formation is represented by the electrostatic interactions. However, recent discussions on the cytochrome *c*-cytochrome *c* peroxidase complex have suggested that the electrostatically stabilized 1 : 1 complex is not optimized for electron transfer and that a decrease in the ionic interactions induced a reorientation of the two proteins which allowed the formation of a more efficient electron-transfer complex [55]. Moreover, Mauk and Mauk [17] have recently proposed that the cross-linking technique would not be the best way to elucidate the intermolecular interface in bimolecular complexes. But, as the overall geometry of the complex is in any case maintained and if the covalent product exhibits an enzymatic activity comparable to the native non-covalent complex, identification of the cross-linked regions provides important information for the understanding of protein–protein interaction and constitutes a very interesting basis for the study of the electron transfer mechanism between two redox proteins. This study allowed us to elucidate the interacting domain between cytochrome c_3 and ferredoxin I and to specify the mechanism of complex formation. This characterization leads us to consider the cytochrome c_3-ferredoxin I complex as a model, since it constitutes the only detailed bimolecular complex between an iron-sulfur cluster and heme-containing proteins. For further understanding of the structure–function relationships in the tetraheme cytochrome c_3 and specification of both intra- and intermolecular electron exchange mechanisms, a site-directed mutagenesis program on this molecule is in progress [56]. Moreover, based on the preparation of cross-linked complexes, we have undertaken to determine the site of flavodoxin and hydrogenase interaction site on cytochrome c_3. A comparison of the reactivity of the different cytochrome electron transfer partners (hydrogenase, flavodoxin) will be investigated using native and covalently ferredoxin-bound cytochromes c_3.

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