Biochimica et Biophysica Acta, 1144 (1993) 125–133

BBABIO 43873

Intramolecular electron transfer in ferredoxin II from *Desulfovibrio desulfuricans* Norway

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(Received 22 January 1993)

Key words: Electron exchange; Intramolecular electron exchange; Ferredoxin II; (*D. desulfuricans* Norway)

In order to elucidate the role of the two (4Fe-4S) clusters in ferredoxins and to determine whether an electron-transfer mechanism may occur between the clusters, the in vitro reduction of cytochrome c_3 and cytochrome c_{553} by *Desulfovibrio desulfuricans* Norway ferredoxin II was studied using spectrophotometric techniques. Ferredoxin II, covalently cross-linked with either cytochrome c_3 or c_{553}, is an obligate intermediate in cytochrome reduction by pyruvate dehydrogenase. Both titration of the complex formation under ^{1}H-NMR spectroscopy and cross-linking experiments between ferredoxin II and either cytochrome c_3 or cytochrome c_{553} gave a stoichiometric ratio of 1:1. Modelling the protein yielded differences between the charge distributions around the two (Fe-S) clusters. The fact that Cluster 2 is blocked in the electron-transfer domain facing the cytochrome interacting heme, indicates Cluster 1 receives electron from pyruvate dehydrogenase. Consecutively, cytochrome reduction occurs owing to an intramolecular electron exchange between the two clusters of the ferredoxin. The properties of two (Fe-S) cluster ferredoxins are compared to those of monocluster ferredoxins and discussed in evolutionary terms.

Introduction

Iron-sulfur proteins are present in many biological systems, such as microorganisms, plants and mammalian cells. (Fe-S) clusters are present in enzymes and in membrane-bound electron-transfer chains, often in complex multisubunit proteins which contain several iron-sulfur clusters [1]. (Fe-S) proteins have been studied using various chemical methods and a large number of physical techniques, such as EPR, Mössbauer spectroscopy, Extended X-ray Absorption Fine Structure (EXAFS) and X-ray crystallography [2–4]. It has been established that fumarate reductase, succinate dehydrogenase [5] and Fe-hydrogenase [6] contain conserved amino-acid sequences which are homologous to two (4Fe-4S) cluster ferredoxins. The information obtained from the smaller structures, such as ferredoxins, provides an invaluable basis for studying the larger molecules belonging to this class of proteins.

In order to understand the electron-transfer mechanism between (Fe-S) clusters in metalloenzymes, electron-exchange processes have to be studied in the two (4Fe-4S) cluster ferredoxins. The question arose as to whether the two clusters play equivalent roles in the molecular reaction processes: the only information available in this connection however is the X-ray structure of *Peptococcus aerogenes* two (4Fe-4S) cluster ferredoxin [7], but little useful data have been deduced from this structure. Although the average distance between the two clusters (12 Å) is in agreement with an intramolecular electron exchange, no structural hypothesis has been proposed on the electron-transfer pathway between the two clusters.

Two ferredoxins have been isolated from *Desulfovibrio desulfuricans* Norway. Ferredoxin I is a monocluster ferredoxin and ferredoxin II contains two (4Fe-4S) clusters. Biophysical experiments on the ferredoxin I/cytochrome c_3 complex formation have shown that one of the hemes of the tetrahemic cytochrome from *D. desulfuricans* Norway, namely heme 4 in the crystal structure, is the heme interacting with ferredoxin in the molecule [8]. The monocluster ferredoxin from the same organism was cross-linked with cytochrome c_3 in an active covalent cross-linked complex. The cross-linked ferredoxin was reduced by hydrogenase under hydrogen atmosphere through an intramolecular electron

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transfer within the tetraheme cytochrome and an intermolecular heme/(Fe-S)-cluster electron exchange. A hypothetical electron-transfer pathway within cytochrome c_3 was suggested [8] according to which aromatic amino acids are the intervening residues. This mechanism might be the general electron pathway pattern in most multiredox center proteins.

D. desulfuricans Norway ferredoxin II is a two (4Fe-4S) cluster ferredoxin similar to *P. aerogenes* ferredoxin [9]. The redox potentials of ferredoxin were around −440 mV and the protein was found to be very unstable to oxygen or to a temperature increase. Numerous homologies exist between the amino-acid sequence of ferredoxins I and II, and the disappearence of one of the two clusters in ferredoxin I is associated with the lack of two cysteine residues [10]. This loss of one of the clusters is compensated for by the emergence of an α helix in the monocluster ferredoxin [11]. Although ferredoxins I and II were found in in vitro pyruvate dehydrogenase reaction or sulfite reduction experiments to have similar activities [12], the presence of the second cluster in ferredoxin II still remains to be explained.

To investigate the intramolecular electron transfer between the two (Fe-S) clusters in ferredoxin, we studied the reactivity of *D. desulfuricans* Norway ferredoxin II covalently bound to a monohemic and a tetrahemic cytochrome. Spectrophotometric measurements of the cytochrome reduction in an in vitro reaction involving ferredoxin and pyruvate dehydrogenase were carried out. A model of *D. desulfuricans* Norway ferredoxin II was built as a mean of interpreting the electron-transfer mechanism in the protein in structural terms.

Materials and Methods

Cytochrome c_3 from *D. desulfuricans* Norway strain (NCIB 8310) [13] and cytochrome c_{553} from *D. vulgaris* Hildenborough strain (NCIB 8303) [14] were purified as previously reported. Ferredoxins I and II were isolated from the periplasmic fraction of *D. desulfuricans* Norway cells as described by Van der Westen et al. [15]. The purification steps were the same as those described in Ref. 12.

1-D ^{1}H-NMR spectra

The protein solutions were buffered with potassium phosphate (pH 7.6). Cytochrome c_3 and c_{553} samples were prepared by performing D_2O exchanges and successive lyophilizations. Ferredoxins I and II were concentrated in D_2O on an Amicon microconcentrator centricon. ^{1}H-NMR spectra were recorded in a Fourier mode on a Brüker AM 200 spectrometer with the water line preirradiated. Chemical shifts are expressed in parts per million (ppm) from internal tetramethyl silane (TMS). Parameters of protein complex formation were determined by calculating the relative induced chemical shifts ($\Delta\delta_{ij}/\Delta\delta_{it}$, where $\Delta\delta_{ij}$ is the induced chemical shift variation and $\Delta\delta_{it}$ is the maximal induced chemical shift variation) as a function of a ratio (ferredoxin II/cytochrome c_3 or c_{553}) as previously reported in the case of the cytochrome c_3-ferredoxin I complex [16]. Parameters of protein complex formation were calculated from chemical shift variations in each cytochrome methyl line affected, on the basis of at least two different experiments.

Covalent cross-linking

Covalent cross-linking was achieved by processing 18 μM cytochrome (cytochrome c_3 or cytochrome c_{553}) and 18 μM ferredoxin (Fd I or Fd II) with 10 mM EDC (*N*-ethyl-3-3-dimethylaminopropyl carbodiimide) for 90 min at room temperature in 10 mM cacodylate buffer (pH 6). The reaction was stopped by adding ammonium acetate at a final concentration of 0.1 M as described by Dolla and Bruschi [17].

Purification of cross-linked complexes

The reaction mixture was chromatographed on an Ultrogel ACA44 (LKB) column (110 cm × 1 cm) equilibrated and eluted with 400 mM NaCl, 50 mM Tris-HCl (pH 7.6) at a flow rate of 10 ml/h. The fractions were analyzed by means of SDS-PAGE.

Electrophoresis was performed on SDS-polyacryamide gels consisting of 13% polyacrylamide running gel and 5% polyacryamide stacking gel. Protein bands were visualized by staining with Coomassie blue R-250.

Amino-acid analysis and sequence determination

Amino-acid analyses were carried out on a Beckman 6 300 amino-acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110°C for 18 h.

Sequence determinations were performed on an Applied Biosystems A 470 gas-phase sequencer with 1 nmol of protein. Quantitative determination of phenylthiohydantoin derivatives was carried out using a HPLC technique (Waters).

Enzyme assays

In vitro reduction of cytochromes (cytochrome c_3 or cytochrome c_{553}) by pyruvate dehydrogenase was monitored spectrophotometrically. The reaction mixture contained 25 μmol phosphate buffer (pH 7), 1.3 μmol coenzyme A, 1.6 μmol thiamin pyrophosphate, 3 μmol β-mercaptoethanol, 3 μmol $MgCl_2$, 5 nmol electron acceptor and the pyruvate dehydrogenase-containing extract in a final volume of 1.0 ml. The pyruvate dehydrogenase-containing extract was depleted of both cytochromes and ferredoxins. After adding 10 μmol sodium pyruvate to the reaction mixture, the cytochrome reduction by pyruvate dehydrogenase was

studied from the increase in the 418.2 nm absorbance under an N_2 atmosphere.

Computer graphics modelling

The three-dimensional model of *D. desulfuricans* Norway ferredoxin II was built on the basis of the chemical sequence and the X-ray structure of the homologous ferredoxin from *P. aerogenes* [7]. Atomic coordinates of the latter were obtained from the Brookhaven Protein Data Bank. Sequence modifications were performed with the TURBO-FRODO program [18] implemented on a Silicon Graphics 4D25GT station. Residues were replaced according to the amino-acid sequence alignment (Fig. 1) and atoms were deleted or introduced using the appropriate stereochemistry from the internal dictionary. The inserted fragments were modelled taking to the most plausible conformation in view of the surrounding environment and using highly-refined three-dimensional structures available in the Protein Data Bank [19]. The model was energy-minimized in the final modelling stage using the molecular dynamics program XPLOR [20].

Results

Electron transfer in cytochrome c_3/ferredoxin II complex

Evidence that a complex is formed between ferricytochrome c_3 and ferredoxin II from *D. desulfuricans* Norway was obtained from the NMR experiments. Titration experiments are illustrated in Fig. 2A in which ferricytochrome c_3 ^{1}H-NMR spectra were recorded with increasing amounts of ferredoxin. *D. desulfuricans* Norway cytochrome c_3 contains four hemes with low redox potentials (−165, −305, −365 and −400 mV) [21]. From the ^{1}H-NMR spectra of cytochrome c_3 in various oxido-reduction states, ring methyl resonances belonging to the same heme group have been detected (Fig. 2A) [22]. The presence of ferredoxin II results in selective modifications in the ferricytochrome c_3 ^{1}H-NMR spectrum, as was reported in the case of the cytochrome c_3-ferredoxin I complex formation [16]. The heme methyl resonances of two hemes, namely I and II, assigned to the two highest redox potential hemes, are shifted in the presence of either ferredoxin I or II. The four ring methyl resonances of the two hemes are not equally severely perturbed. Two ring methyl lines of heme I (13.81 and 14.42 ppm) and two ring methyl lines of heme II (14.82 and 21.35 ppm) are significantly affected by the presence of ferredoxin II. The relative induced shifts ($\Delta\delta_{ij}/\Delta\delta_{it}$) computed at low ionic strengths as a function of the ferredoxin II/cytochrome c_3 ratio (Fig. 2B) showed that a 1:1 complex had formed between one molecule of cytochrome c_3 and one molecule of ferredoxin II. The increase in the ionic strength induced a decrease in the association constant, in agreement with the strong electrostatic effect on the complex formation previously observed in the case of the cytochrome c_3/ferredoxin I complex [23].

A covalent cross-linked complex was prepared as described by Dolla and Bruschi [17]. On the basis of the NMR data on the cytochrome c_3/ferredoxin II complex formation, cytochrome c_3 (17500 Da) and ferredoxin II (6000 Da) were incubated with EDC in a 1:1 ratio. SDS-PAGE of the reaction mixture showed the formation of two new compounds with apparent molecular masses of 40 and 24 kDa, respectively (Fig. 3, lanes 5 and 6). The lowest band corresponds to the main reaction product. Minor amounts of free cytochrome and free ferredoxin were observable (Fig. 3, lanes 5 and 6). Incubating cytochrome c_3 with EDC does not induce the formation of polymeric cross-linked products (Fig. 3, lane 4), but does so in the case of ferredoxins I and II (Fig. 3, lane 3). The two compounds were separated by gel filtration on an Ultrogel ACA44 column at high ionic strength to prevent any electrostatic interactions. Amino-acid analysis and N-terminal sequence determination (ADAPG with cytochrome c_3 and MGYSV with ferredoxin II, found to be present in similar amounts) were performed on the two fractions, which were identified as cytochrome c_3/ferredoxin II complexes with a stoichiometric ratio of 1:1. The higher-molecular-mass complex is a dimer of the lower one, probably due to dimerisation of the ferredoxin.

A bidirectional electron transfer between ferredoxin and cytochrome c_3 has already been found on the basis

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                1         10        20        30        40
FdPa            AYVINDSCIACGACKPECPVN---IQQGSIYAIDADSCIDCGSC
Fd2DdN    MGYSVIVDSDKCIGCGECVDVCPVEVYELQNGKAVPVNEEECLGCESC
          1         10        20        30        40

                50
FdPa      ASVCPVGAPNPEP
Fd2DdN    IEVCPQNAI-VE
          50
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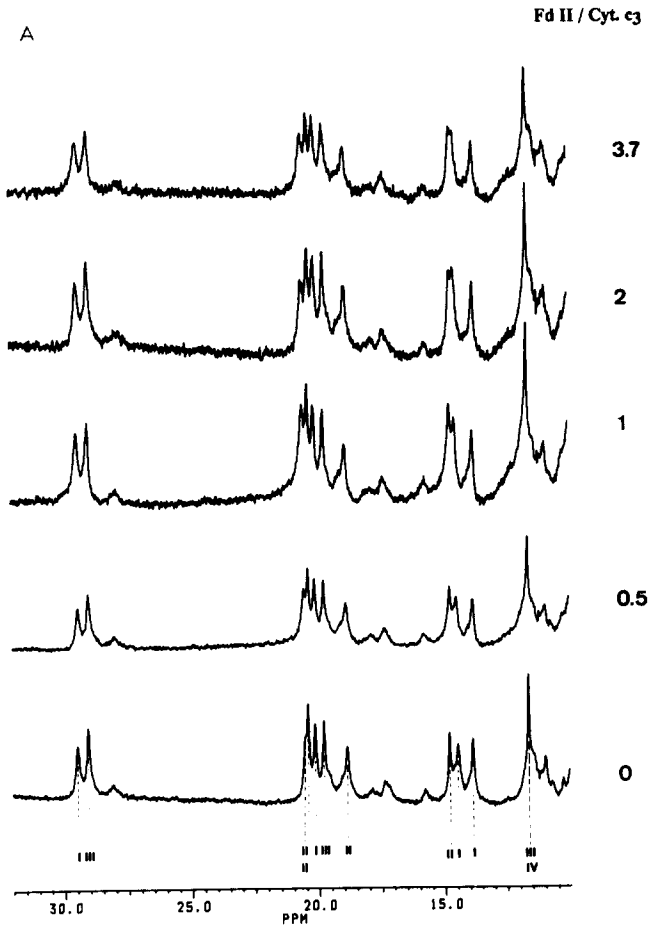
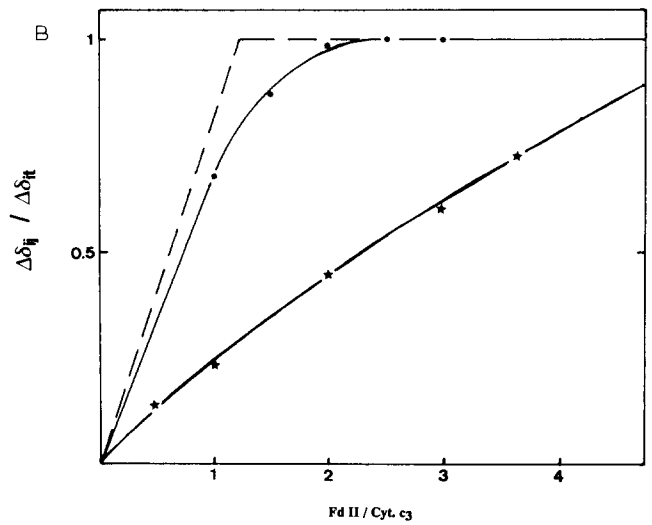
Fig. 1. Amino-acid sequence alignment of ferredoxins from *P. aerogenes* (Fd Pa) and *D. desulfuricans* Norway (Fd2 DdN) based on Hydrophobic Cluster Analysis [36].

of in vitro measurements to occur in both the phosphoroclastic and sulfite reduction reactions [12]. Ferredoxin is essential for coupling either pyruvate dehydrogenase or sulfite reductase to cytochrome c_3. Fig. 4, bar a, shows that in the absence of ferredoxin, no





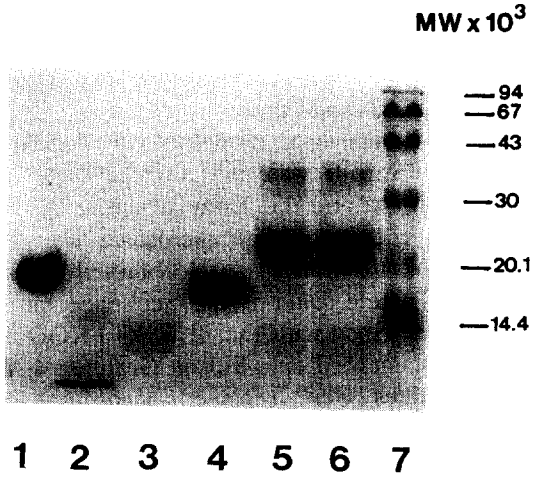


Fig. 3. SDS-PAGE of covalent cross-linking between cytochrome c_3 and ferredoxin II. lane 1, cytochrome c_3; lane 2, ferredoxin II; lane 3, ferredoxin II treated with EDC; lane 4, cytochrome c_3 treated with EDC; lanes 5 and 6, cytochrome c_3 incubated with ferredoxin II and EDC for 45 and 90 min, respectively; lane 7, standard proteins with the following molecular masses: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

increase occurs in the cytochrome absorption at 418.2 nm, while in the presence of either ferredoxin I or ferredoxin II in equimolar concentrations, 100% of the cytochrome c_3 is reduced (Fig. 4, bars b and c). The covalent cross-linked cytochrome c_3 is reduced when it is bound to ferredoxin II (78%) but not when it is bound to ferredoxin I (5%) (Fig. 4, bars d and e). The absence of reduction by the covalently bound monocluster ferredoxin has been previously reported by Dolla and Bruschi [17] and interpreted as resulting from the inaccessibility of the (Fe-S) cluster which abolishes the electron transfer from pyruvate dehydrogenase to ferredoxin. Due to the presence of the second cluster in ferredoxin II, the reduction of the cytochrome occurs through an intramolecular electron exchange in two-cluster ferredoxin. However, the possibility that one of the other hemes of the tetrahemic

Fig. 2. (A) ^{1}H-NMR spectra of ferricytochrome c_3 in the presence of an increasing ferredoxin II/cytochrome c_3 ratio. The spectra were recorded at 20°C and only the 32 to 11 ppm region is shown. Roman numerals indicate the assignment of the heme methyl lines to the four hemes depending on their oxido-reduction potential (I, −165 mV; II, −305 mV; III, −365 mV and IV, −400 mV). The ferricytochrome c_3 concentration was 1 mM, pH 7.6 in 100 mM NaCl, 50 mM phosphate buffer. (B) Titration curves plotted from relative shifts ($\Delta\delta_{ij}/\Delta\delta_{it}$) giving the changes in the resonance at 14.82 ppm of the ferricytochrome c_3 ^{1}H-NMR spectrum in the presence of increasing concentrations of the ferredoxin II described in Fig. 2A. ($\Delta\delta_{jj}/\Delta\delta_{it} = (\delta_i$ partially complexed $- \delta_i$ free)/(δ_i completely complexed $- \delta_i$ free) (*) 50 mM NaCl, 5 mM phosphate buffer; (⋆) 100 mM NaCl, 50 mM phosphate buffer.

cytochrome may react with the accessible (Fe-S) cluster of ferredoxin II belonging to another complex prevented us from reaching any definite conclusions as to the mechanism involved in the electron-transfer process in the cytochrome c_3/ferredoxin II complex. In order to provide clear evidence that an intramolecular electron transfer takes place in two (Fe-S) cluster ferredoxin, we carried out a further series of experiments using the monohemic cytochrome c_{553} from *D. vulgaris* Hildenborough.

Electron transfer in the cytochrome c_{553}/ferredoxin II complex

Fig. 5A gives the results of the NMR titration of the complex formation between the monohemic ferricytochrome c_{553} with either *D. desulfuricans* Norway ferredoxin I or ferredoxin II. The ^{1}H-NMR spectrum of ferricytochrome c_{553} shows four clearly-separated resonances in an extremely low field region. These were assigned to ring methyl groups 8, 3, 5 and 1 (from low to high field) of the heme c [24]. The presence of ferredoxin I or ferredoxin II induced modifications in the ^{1}H-NMR spectrum of ferricytochrome c_{553} (Fig. 5B). The resonances corresponding to methyl 5 underwent significant changes. The relative induced shift $(\Delta\delta_{ij}/\Delta\delta_{it})$ computed as a function of the ferredoxin I or II/cytochrome c_{553} ratio showed the existence of 1:1 complexes. With one or two-cluster ferredoxin, the complex formation leads to a stoichiometry of one molecule of cytochrome c_{553} per molecule of ferredoxin. In the case of the ferredoxin II, one might

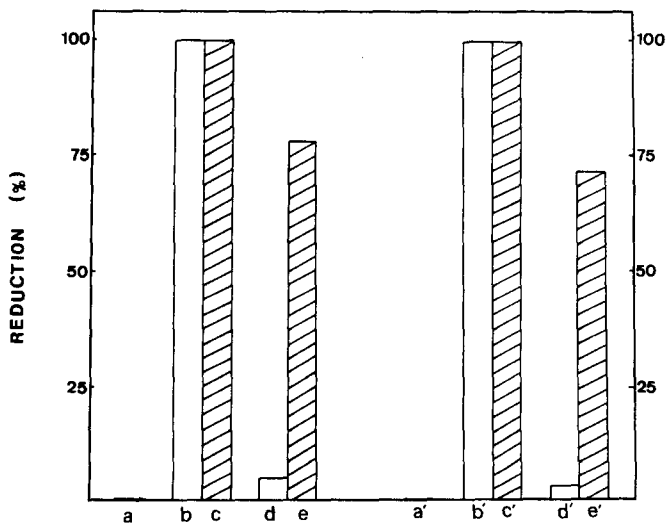


Fig. 4. Cytochrome c_3 and cytochrome c_{553} reduction by pyruvate dehydrogenase. The cuvette (light path width 1 cm) contained the reaction mixture (see Materials and Methods) including varying electron acceptors: a, cytochrome c_3; b and c, cytochrome c_3 in an equimolar mixture with ferredoxin I from Dolla and Bruschi [17] and ferredoxin II, respectively; d and e, The 1:1 covalent complex between cytochrome c_3 and ferredoxin I and that between cytochrome c_3 and ferredoxin II. a', cytochrome c_{553}; b' and c', cytochrome c_{553} in an equimolar mixture with ferredoxin I and ferredoxin II, respectively; d' and e', the 1:1 covalent complex between cytochrome c_{553} and either ferredoxin I or ferredoxin II.

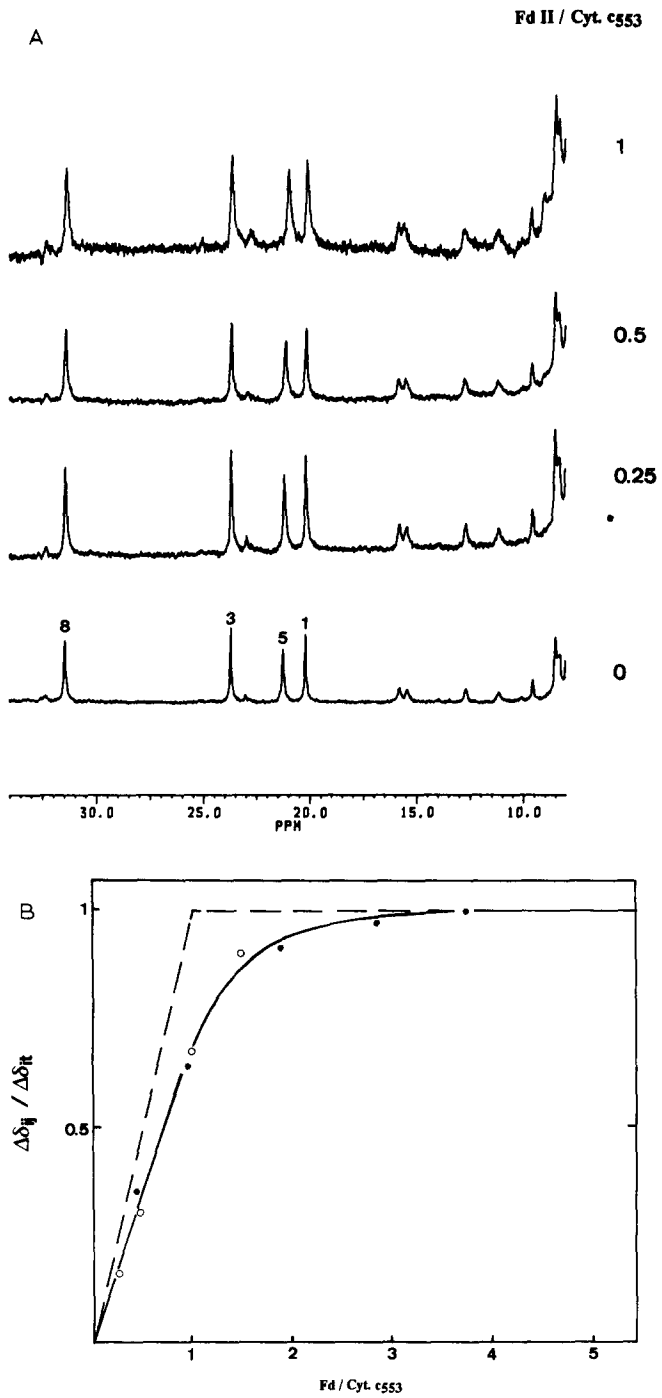


Fig. 5. (A) ^{1}H-NMR spectra of ferricytochrome c_{553} in the presence of an increasing ferredoxin II/cytochrome c_{553} ratio. The spectra were recorded at 20°C and only the 35 to 8 ppm region is shown (the heme methyl lines are denoted by 8, 3, 5 and 1). The ferricytochrome c_{553} concentration was 1 mM, pH 7.6 in 50 mM NaCl, 5 mM phosphate buffer. (B) Titration curves plotted from changes in the shifts of the 5 methyl ring group resonance of the ferricytochrome c_{553} ^{1}H-NMR spectrum, in the presence of increasing concentrations of ferredoxin I (*) or ferredoxin II (○). $(\Delta\delta_{ij}/\Delta\delta_{it} = (\delta_i$ partially complexed $- \delta_i$ free$)/(\delta_i$ completely complexed $- \delta_i$ free)).

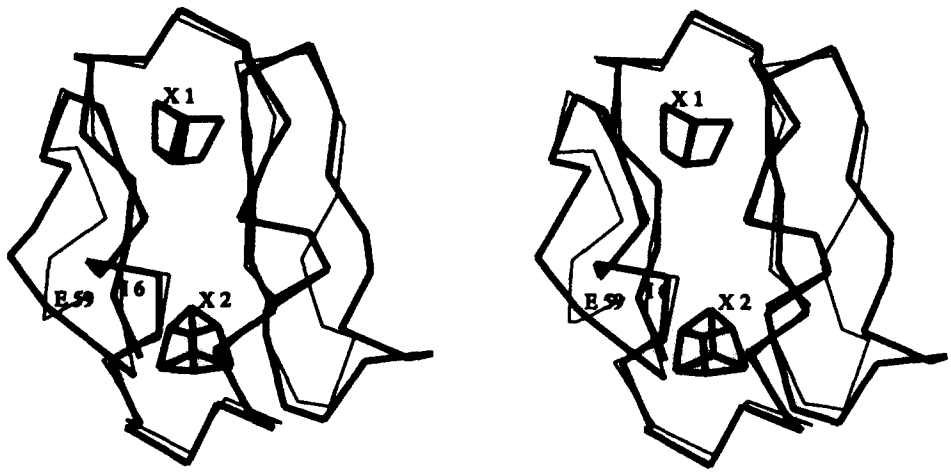


Fig. 6. A stereo view of the α-carbon backbone of *P. aerogenes* ferredoxin (thin lines) superimposed on that of *D. desulfuricans* Norway ferredoxin II (thick lines). The exact geometry and relative positions of the two (Fe-S) clusters, X1 and X2 observed in *P. aerogenes* ferredoxin were strictly modelling the *D. desulfuricans* Norway ferredoxin II. The two structures differed significantly in the loop region containing residues 23–30.

expect two cytochrome molecules to be associated per ferredoxin molecule. These results suggest that the two clusters (1 and 2) in ferredoxin II are not both involved in the cytochrome interacting domain.

Incubating cytochrome c_{553} (9 kDa) and ferredoxin I or II (6 kDa) in an equimolar mixture with EDC led to the formation of a main compound with a molecular mass of about 15 kDa (data not shown). This main cross-linked product was purified by gel filtration. The results of amino-acid analysis and N-terminal sequence determination (ADGAA with cytochrome c_{553}, MGYSV with ferredoxin II and TIVID with ferredoxin I were detected in similar amounts) demonstrated that the new compound was a cytochrome c_{553}-ferredoxin II or cytochrome c_{553}/ferredoxin I complex with a stoichiometric ratio of 1 : 1.

The occurrence of an intramolecular electron-transfer process within ferredoxin II was demonstrated by coupling pyruvate dehydrogenase to the cytochrome c_{553}/ferredoxin II covalent complex. A 70% reduction of the cytochrome c_{553} was observed when it was cross-linked with ferredoxin II, as compared to an equimolar solution of the two proteins (Fig. 4, bar e′) and a 3% reduction when it was covalently bound to the monocluster ferredoxin (Fig. 4, bar d′). The presence of free ferredoxin I leads, however, to coupling between pyruvate dehydrogenase and cytochrome c_{553} (Fig. 4b′). The reduction of cytochrome c_{553} covalently bound to ferredoxin, therefore, necessitates the presence of a second (Fe-S) cluster in the ferredoxin. One of the clusters is involved in the cytochrome interacting domain and the second is accessible to the pyruvate

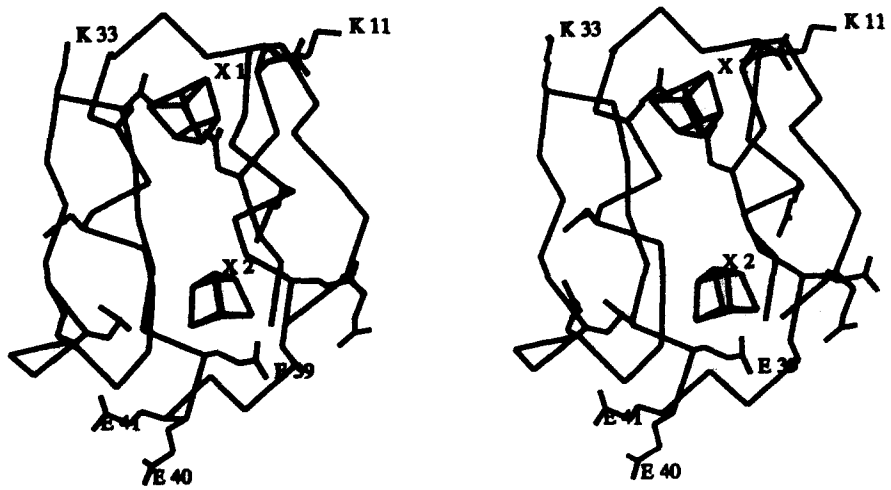


Fig. 7. Stereo view of the two cluster poles: one pole (cluster 1) mainly consisted on two aspartic (D8 and D10) and two lysine residues (K11 and K33), while the second (cluster 2) consisted of a larger amount of acidic residues, especially E39, E40 and E41.

dehydrogenase. The intramolecular electron exchange within ferredoxin II allows to connect the cytochrome and the pyruvate dehydrogenase interacting clusters.

The next session is to give evidence which cluster (1 or 2) in ferredoxin II is the cytochrome interacting site.

Model of D. desulfuricans Norway ferredoxin II

On the basis of the *P. aerogenes* ferredoxin structure and sequence alignment (Fig. 1), a three-dimensional model of *D. desulfuricans* Norway ferredoxin II was built. The alignment shows twenty one strictly conserved residues, three insertions and one deletion. Superimposing *D. desulfuricans* Norway on the *P. aerogenes* ferredoxin structure indicated that the main chain folding of the molecule was highly conserved except within the loop 23–30. Lastly, the model was subjected to an energy-minimization procedure and found to be very similar in this respect to the reference structure (concerning 42 Ca positions, the maximum deviation is 1.0 Å and the r.m.s. value is 0.53 Å, for the 55 Ca, the maximum deviation is 4.9 Å giving a r.m.s. value of 1.35 Å) (Fig. 6). The 2-fold symmetry observed in the tertiary structure of *P. aerogenes* ferredoxin was therefore also conserved in the ferredoxin II model. In the latter model however, the environments of the two (Fe-S) clusters differed significantly with regard to the charge distribution. The two lysine residues (Lys-11 and Lys-33) of the sequence and two aspartic chains (Asp-8 and Asp-10), were found in the vicinity of cluster 1, whereas cluster 2 was surrounded by five glutamic residues (Glu-25, 39, 40, 41 and 46) (Fig. 7).

It is generally assumed that electrostatic interactions are the driving force in the formation of electron-transfer precursor complexes. In this respect, the ring of acidic residues around cluster 2 may be an ideal structural feature for electrostatic coupling with basic side chains of the cytochrome c_3 redox partner.

A similar approach was applied to *D. vulgaris* Miyazaki ferredoxin I (data not shown) and indicated that this two-cluster ferredoxin contains a ring of acidic residues around each of the two clusters. This finding explains the stoichiometric ratio of one ferredoxin I molecule per two cytochrome c_3 molecules obtained by Park et al. [25].

Discussion

It was established in the present study on the basis of NMR data and cross-linking experiments that a complex is formed between either cytochromes c_{553} or c_3 and either ferredoxins I or II at a stoichiometric ratio of 1:1, despite the presence of one or two clusters in the ferredoxin and the presence of one or four hemes in the cytochrome. These results indicate that one heme has a particular affinity for one (Fe-S) cluster. The model of the cytochrome c_3-ferredoxin I complex, supported by the results of the cross-linking experiments, demonstrated that heme 4 (sequence numbering) is the reactive heme in cytochrome c_3 [8,26]. In the case of the two (Fe-S) cluster ferredoxin we demonstrate that one of the two (Fe-S) clusters is involved in the cytochrome interacting domain, our model of the ferredoxin II structure shows that a ring of glutamic acids surrounds cluster 2 while the two aspartic acids present around cluster 1 are neutralized by the two lysine residues of the molecule. Cluster 2 is therefore probably the (Fe-S) cluster interacting with cytochrome in ferredoxin II. It is worth noting that in the monocluster ferredoxin, the conserved cluster is cluster 1 in which acidic residues are conserved and that K11 is replaced by a glutamic acid. The following question now arises: does the second cluster in ferredoxin II play only a structural role or is there a physiological explanation for this multiredox protein? Ferredoxins have been described as proteins having a broad range of activities: their involvement in other hitherto undescribed reactions would explain the presence of the two different ferredoxins in the *D. desulfuricans* Norway strain. Moreover, our experiments involved reconstituted systems and no information is available so far on the in vivo activity of these ferredoxins.

Fukuyama et al. [27] have proposed a model for the evolution of ferredoxins, according to which the one cluster ferredoxin from *Bacillus thermoproteolyticus* and the two cluster ferredoxin from *P. aerogenes* evolved from a common ancestor. The clostridial-type ferredoxins conserve the basis of the duplicated ancestral sequence together with the 2-fold symmetry in the tertiary structure. In monocluster ferredoxin, the regions corresponding to the four cysteine residues necessary to bind the lost cluster are replaced by the helical structure observed in the (4Fe-4S) cluster ferredoxin from *D. desulfuricans* Norway [11]. This helix probably plays a role in maintaining the tertiary structure.

It might be speculated that the evolution of the ancestral ferredoxin may have led to two different proteins in *D. desulfuricans* Norway:

(i) the two (4Fe-4S) cluster ferredoxin II: the two clusters have acquired different specificities, due to the differences in the charge distribution around the two (Fe-S) clusters.

(ii) the (4Fe-4S) ferredoxin I: cluster 2 has disappeared, along with two cysteinyl residues.

Various intermediates in the course of ferredoxin evolution are to be found, however, in other *Desulfovibrio* species, such as the 7 Fe-ferredoxin of *D. vulgaris* Miyazaki or *D. africanus* [28].

The present study provides evidence that an intramolecular electron transfer occurs in two-cluster ferredoxins. During our experiments we observed that cytochrome reduction is less efficient when cross-linked

ferredoxin II is involved than in the non-covalent complexes. One explanation might be that ferredoxin II contains only one reactive (Fe-S) cluster (cluster 2), and that this site is blocked in the covalent cross-linked complex facing the heme of the cytochrome. Cluster 1 would then also have a minor affinity for pyruvate dehydrogenase and the electron-transfer process would therefore be less productive.

Although two dinuclear ferredoxin structures have been elucidated [7,29], no explanations have been put forward so far concerning a hypothetical intramolecular electron-transfer pathway. We previously suggested that aromatic residues might play a crucial role in this pathway in multi-redox proteins [30]. In ferredoxins it has been observed that the clusters exist in quite hydrophobic environments and it was suggested that conserved aromatic rings might in fact play an important part in electron transfer [31]. If one compares the ferredoxin II model with the *P. aerogenes* ferredoxin structure, it can be seen that the tyrosine (Tyr-3) located in the N-terminal region is conserved while the second tyrosine (Tyr-27) is considerably shifted, as it is included in the most greatly-modified loop (Figs. 1 and 7B). This structural information underlines the asymmetry of ferredoxin II as compared to *P. aerogenes* ferredoxin, and shows that cluster 2 is involved in the electron-transfer mechanism in the protein. EPR experiments have suggested that a paramagnetic interaction occurs between the two clusters [32]. In view of this paramagnetic interaction, a direct coupling may exist between the two clusters. Up to now, the crystallographic structures of two metalloenzymes have been determined which may provide a basis for a possible electron flow mechanism between the redox centers: one, trimethylamine dehydrogenase (TMAD), is a (4Fe-4S) flavoprotein, the structure of which was resolved at 2.4 Å [33]; the spin-coupling model based on EPR studies predicts that the two centers are in close proximity, which is consistent with the distance of only 4 Å observed in the crystal structure. The other known crystallographic structure is that of sulfite reductase [34]; the siroheme and the (4Fe-4S) cluster are packed against each other (5.5 Å) and they were found to share a common ligand. It should be mentioned that the closest distance between the two clusters in ferredoxin II (cluster 1 Fe_4 and cluster 2 S_3) was 8.85 Å. This structural feature is still consistent with the existence of a direct electron-transfer pathway between the two prosthetic groups. Site-directed mutagenesis studies would now be useful to definitely explain the physiological role played by this mechanism involving the two (Fe-S) clusters in ferredoxins. The synthetic gene of ferredoxin II has been cloned in *Escherichia coli* and the functional expression of the protein is currently being studied by our group [35].

Acknowledgements

The authors wish to thank Dr. A. Dolla for helpful discussions. They are grateful to J. Bonicel for the N-terminal sequence determinations, N. Zylber for amino-acid analysis and the Laboratoire de Chimie Bactérienne Fermentation Unit for producing the microorganisms.

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