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The cytochrome c_3-ferredoxin electron transfer complex: cross-linking studies

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The tetrahemic cytochrome c_3 (M_r = 13000) and the ferredoxin I isolated from *Desulfovibrio desulfuricans* Norway are specific physiological partners in the sulfate-reducing bacteria electron transfer chain. The study of their interaction should improve the understanding of the electron transfer mechanism between heme and iron sulfur clusters. Incubation of cytochrome c_3 and ferredoxin I together with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate allows the formation of a covalently cross-linked complex with a stoichiometry of one cytochrome c_3 molecule for one ferredoxin I subunit. The ionic strength dependence of the cross-linking and the absence of a covalent reaction when ferredoxin is replaced by another acidic protein suggest that this covalent complex is a valid model of the native non-covalent complex. The study of its physiological activities indicates the existence of one or several other interacting sites on cross-linked cytochrome and would explain the role of the multihemic cytochrome.

Introduction

The mechanism by which electrons are transferred between electron carrier proteins in oxidative phosphorylation and photosynthesis has been reported to involve direct interaction between the prosthetic group of the corresponding oxidoreductases. Formation of intermediate complexes between *c*-type cytochrome and its oxidoreductases has been illustrated by the crystallographic structure of a bacterial photosynthetic reaction center [1] and by hypothetical models of electron transfer complexes based on structural data [2–4]. The study of these complexes by NMR, chemical modifications [5] or chemical cross-linking [6] are in agreement with the proposed models.

As *Desulfovibrio desulfuricans* Norway cytochrome c_3 (M_r = 13000) is a tetrahemic protein, the three-dimensional structure of which is known [7,8], and its physiological partners are iron sulfur proteins like hydrogenase and ferredoxin, the cytochrome c_3-ferredoxin complex is a model for the study of electron transfer reactions involving heme and iron sulfur cluster.

Each heme of cytochrome c_3 exhibits a distinct redox potential (−165 mV, −305 mV, −365 mV and −400 mV) [9,10]. The primary structure of the ferredoxin has been determined [11] and the obtainment of crystals has led to preliminary crystallographic data [12]. Ferredoxin I is an acidic dimeric protein containing one (4Fe-4S) cluster per subunit with a midpoint reduction potential of −374 mV [13].

Cytochrome c_3 and ferredoxin I act as obligate partners in the two metabolic pathways shown in Fig. 1 [14]. Rapid kinetic studies have established a bidirectional electron exchange between the two

Abbrevation: CME-CDI, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate.

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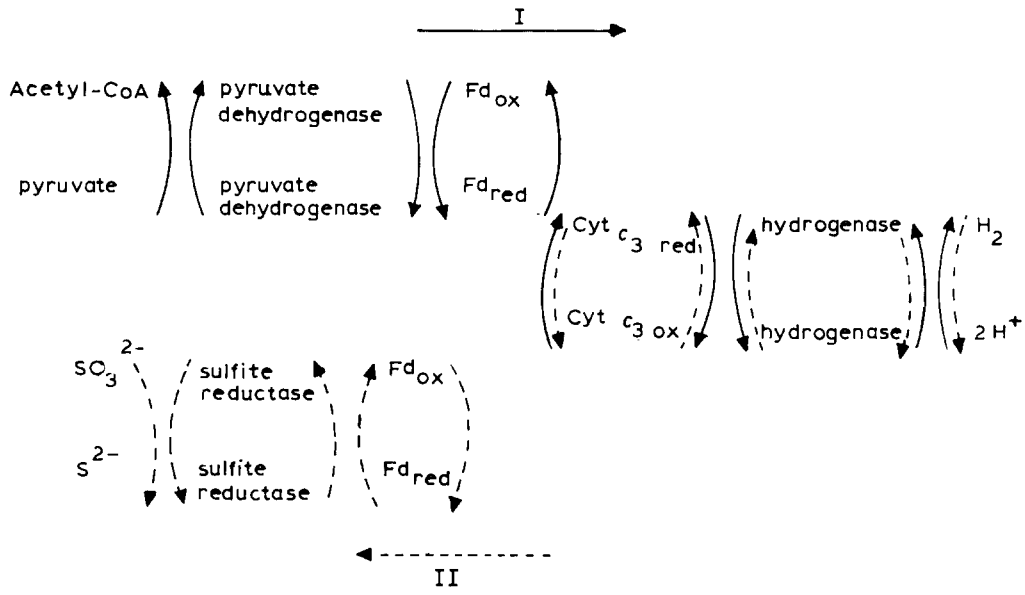

Fig. 1. Electron transport chain from *D. desulfuricans*. (I) The phosphoroclastic reaction and (II) The sulfite reduction.

proteins [15]. Microcalorimetric measurements show an association constant, $K_a = 1.2 \cdot 10^6$ M^{-1} (pH 7.7, 10^{-2} M Tris-HCl buffer, 10 °C), which decreases with increasing ionic strength (Guerlesquin, F., personal communication). These results are interpreted as providing evidence for an electrostatically stabilized complex between the positively charged cytochrome and the negatively charged ferredoxin. From ^{1}H-NMR experiments [16,17], the complex formation stoichiometry was found to be one molecule of cytochrome c_3 and one subunit of ferredoxin. The resonances of only two hemes (−165 mV and −305 mV) are affected by the presence of ferredoxin and one binding site has been found. However, as the four redox potentials are not assigned in the three-dimensional structure, it is not yet possible to identify the heme(s) involved in the binding site.

In an attempt to investigate this recognition site, we have initiated series of cross-linking experiments and we have compared the reduction of cytochrome c_3 in the covalent and native complexes in the two physiological reactions in which these proteins act as obligate intermediates.

Methods

Cytochrome c_3 ($M_r = 13000$), cytochrome c_3 ($M_r = 26000$), ferredoxin I ($M_r = 6000$) and hydrogenase were purified from *D. desulfuricans* Norway as previously described [18–20]. CME-CDI was obtained from Sigma.

Visible and ultraviolet absorption spectra were determined with a PU 8820 spectrophotometer Philips. Protein concentrations were calculated using absorption coefficients of $\epsilon_{552} = 130500$ $M^{-1} \cdot cm^{-1}$ and $\epsilon_{552} = 245200$ $M^{-1} \cdot cm^{-1}$ for reduced cytochromes c_3 $M_r = 13000$ and $M_r = 26000$, respectively, and $\epsilon_{390} = 17500$ $M^{-1} \cdot cm^{-1}$ for oxidized ferredoxin I. The hydrogenase concentration was obtained from amino acid composition analysis.

Covalent cross-linking. Covalent cross-linking was performed by treatment of 18 μM cytochrome c_3 ($M_r = 13000$) and 18 μM ferredoxin I in 10 mM cacodylate buffer (pH 6.0) with 10 mM CME-CDI for 90 min at room temperature. The reaction was stopped by adding ammonium acetate to 0.1 M final concentration.

SDS-polyacrylamide gel electrophoresis. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gels were performed according to Lugtenberg et al. [21] with 13% polyacrylamide running gel and 3% polyacrylamide stacking gel. Protein bands were visualized by staining with Coomassie blue R250 or 3,3′,5,5′-tetramethylbenzidine for heme as described by Trumpower and Aspandiar [22].

Purification of covalent complexes. The reaction mixture was chromatographed on a column (110 cm × 1 cm) of Sephadex G75 equilibrated and eluted with Tris-HCl 0.05 M, NaCl 0.5 M buffer (pH 7.6) at a flow rate of 12 ml/h, 0.5 ml by tube. The fractions were dialyzed overnight at 4° C and analyzed by SDS-polyacrylamide gel electrophoresis.

Isoelectric point. The isoelectric point was determined by isoelectric focusing [23] and an LKB multiphor apparatus.

Amino acid analysis. Amino acid analysis were performed on an LKB 4150 amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110° C for 20 h according to the method of Moore and Stein [24].

Removal of hemes. Apoproteins were obtained by incubating the complexes (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. The reaction mixture was chromatographed on a column of Sephadex G25 equilibrated and eluted with 5% formic acid.

Sequence determination. Sequence determinations were performed in an Applied Biosystems A470 gas-phase sequencer with 1 nmol of apoprotein. The quantitative determination of phenylthiohydantoin derivates was compared to known amounts of appropriate standards by HPLC (high-pressure liquid chromatography, Waters) as described by Bonicel et al. [25].

Enzyme assays. The reaction between the covalent complex and reductases was assayed by measuring spectrophotometrically the cytochrome c_3 reduction.

Reduction of cytochrome c_3 by pyruvate dehydrogenase was studied from the increase of the 418.2 nm absorbance ($A_{418.2}$) under an N_2 atmosphere after adding 10 μmol sodium pyruvate to the reaction mixture which contained: 25 μmol phosphate buffer (pH 7.0), 1.3 μmol coenzyme A, 1.6 μmol thiamin pyrophosphate, 3 μmol $MgCl_2$, 3 μmol β-mercaptoethanol, 5 nmol electron acceptor and the pyruvate dehydrogenase-containing extract in a final vol. of 1.0 ml. The pyruvate dehydrogenase-containing extract, depleted of cytochrome c_3 and ferredoxin I, was obtained by passing *D. desulfuricans* Norway crude extract on a small silica gel column equilibrated with 0.01 M Tris-HCl buffer (pH 7.6) and on a DEAE column equilibrated with 0.1 M Tris-HCl buffer (pH 7.6). For each test, a concentration of 0.51 mg of total protein of this extract was used. After the addition of sodium pyruvate, the equilibrium state was rapidly reached and the $A_{418.2}$ was measured.

Reduction of cytochrome c_3 by hydrogenase was studied from the increase of the 552-nm absorbance (A_{552}) under an H_2 atmosphere when pure hydrogenase was added to the reaction mixture which contained: 50 μmol Tris-HCl buffer (pH 7.6) and 2 nmol electron acceptor. After the addition of 0.46 μg of pure hydrogenase, the equilibrium state was rapidly reached and the A_{552} was measured. The specific activity of hydrogenase was 678 μmol hydrogen consumed per min per mg protein.

Results

Carbodiimide induces cross-linking of ferredoxin I to cytochrome c_3

When cytochrome c_3 ($M_r = 13000$) and ferredoxin I ($M_r = 6000$) are treated with CME-CDI, analysis by SDS-polyacrylamide gel electrophoresis of the reaction mixture shows the formation of two new compounds of apparent $M_r = 38500$ and $M_r = 24000$ (Fig. 2, lane 6). This last complex constitutes the main reaction product; the former is obtained at very low yield. At a different temperature (0° C), another protein concentration (180 μM) or greater carbodiimide molar excess a smaller compound ratio of $M_r = 24000$ is induced, which is obtained in the highest yield under the conditions described in Methods. The incubation of cytochrome c_3 alone or ferredoxin I alone with CME-CDI does not induce the formation of polymeric cross-linked products. Staining with tetramethylbenzidine, specific to the heme-containing peptides, show that each new compound contains hemes.

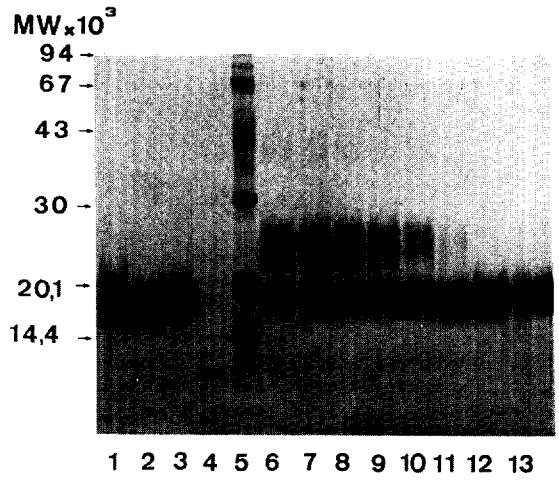

Fig. 2. Analysis of covalent cross-linked species of cytochrome c_3·ferredoxin I. Shown are SDS-polyacrylamide gels of cytochrome c_3 (M_r = 13000) (18 μM) incubated either with ferredoxin I (18 μM, lanes 6–13) at inceasing ionic strength (the NaCl concentrations were (M): 0, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1, respectively) or cytochrome c_3 (M_r = 26000) (18 μM, lane 2), plus CME-CDI (10 mM), as described under Methods. Controls: lane 1 cytochrome c_3 (M_r = 26000); lane 3, cytochrome c_3 (M_r 13000); lane 4, ferredoxin I; lane 5, molecular weight standard proteins: phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), α-lactalbumin (14400).

Purification and molecular properties of the cross-linked complexes

The covalent complex of M_r = 24000 is purified by gel filtration on a Sephadex G75 column at high ionic strength to prevent non covalent interactions.

As cytochrome c_3 has an M_r of 17500 by SDS-polyacrylamide gel electrophoresis, the molecular weight of this complex seems to be the sum of one cytochrome c_3 molecule and one ferredoxin I subunit. The comparison of the cytochrome and ferredoxin equimolar mixture and the covalent complex amino acid analysis is in agreement with the 1 : 1 stoichiometry. Moreover, the N-terminal sequence determination points out the linkage of one ferredoxin I chain for one cytochrome c_3 chain. If the cross-linking reaction is performed with increasing ionic strength, the yield of covalent complexes decreases and no covalent complex is formed when the NaCl concentration is as much as 0.2 M (Fig. 2, lanes 7–13). This result is in agreement with the microcalorimetric data, assuming that the specific complex formation is governed by the establishment of ionic pairs. The incubation of cytochrome c_3 (M_r = 13000) with cytochrome c_3 (M_r = 26000) (an acidic protein isolated from the same microorganism which has not been described as one of its physiological partners) instead of ferredoxin under the same conditions, does not induce the formation of new compounds of greater molecular weight (Fig. 2, lane 2). This suggests that the conditions used do not allow the covalent cross-linking of unspecific complex.

The spectrum of the purified 1 : 1 covalent complex does not show any difference with the cytochrome and ferredoxin equimolar mixture spectrum (Fig. 3). The spectrum shape suggests that there is no perturbation of the hemes due to the cross-linking reaction. The comparison of the re-

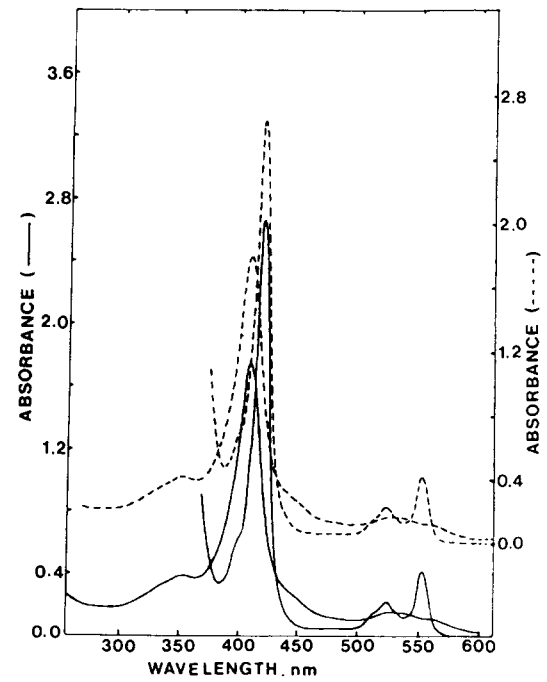

Fig. 3. Absorption spectra of the cytochrome c_3-ferredoxin I covalent complex and of the cytochrome c_3 and ferredoxin I equimolar mixture in oxidized and dithionite reduced states. Covalent complex ($3.2 \cdot 10^{-6}$ M) oxidized and reduced spectra (———); equimolar mixture ($3.2 \cdot 10^{-6}$ M) oxidized and reduced spectra (------).

duction with disodium dithionite of the 1 : 1 covalent complex and the equimolar mixture of cytochrome and ferredoxin show that cross-linked cytochrome c_3 is fully reduced. The ratio of the absorbance of reduced complex at 552 nm, specific to heme *c*, to the 280-nm absorbance, specific to protein, is in agreement with a 1 : 1 complex of cytochrome c_3 and ferredoxin I. Further insight comes from the isoelectric pattern of the 1 : 1 covalent complex. A fuzzy band focuses from $pI = 4.7$ to $pI = 5.9$. These values are intermediates between the cytochrome c_3 pI ($pI = 7$) and the ferredoxin I pI ($pI = 3.9$). Another fuzzy band shows an isoelectric point between 6.5 and 7.2. This isoelectric pattern may be interpreted as a heterogeneity of the number and sites of cross-linking in the covalent molecules population.

Cytochrome c_3 and ferredoxin I covalent complex retains electron transfer activities

In order to test the physiological activities of the covalent complex, two different assays were performed. As cytochrome c_3 ($M_r = 13000$) and ferredoxin I interfere in phosphoroclastic reaction and sulfite reduction (Fig. 1) with a bidirectional electron transfer, the reduction of cytochrome c_3 by pyruvate dehydrogenase and hydrogenase was measured. The percentage of cytochrome c_3 reduction was obtained by comparison with the disodium dithionite reduction. As the rates of electron exchange are very fast [15], the measurement of the cytochrome c_3 reduction was performed at equilibrium.

Reduction by pyruvate dehydrogenase

When sodium pyruvate is added to the reaction mixture containing cytochrome c_3 as electron acceptor, there is a slight increase of the $A_{418.2}$ which corresponds to 0.39% of cytochrome c_3 reduction (Fig. 4A). On the other hand, when electron acceptors are a cytochrome and ferredoxin equimolar mixture, cytochrome c_3 is fully reduced (Fig. 4B). This experiment shows that ferredoxin I is necessary for the electron exchange between pyruvate dehydrogenase and cytochrome c_3. The slight increase of the $A_{418.2}$ observed when cytochrome c_3 alone is the electron acceptor is probably due to a small amount of ferredoxin in the pyruvate dehydrogenase-containing extract. A

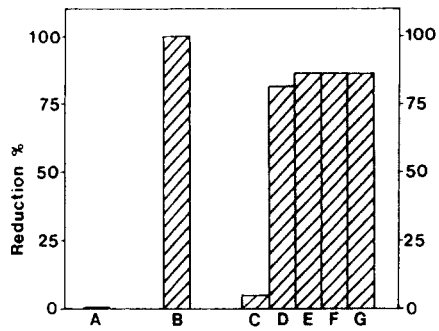

Fig. 4. Reduction of cytochrome c_3 by pyruvate dehydrogenase. The cuvette (1-cm path light) contains the reaction mixture (see Methods) including varying electron acceptors: A, cytochrome c_3 ($M_r = 13000$); B, the cytochrome and ferredoxin equimolar mixture; C, the 1 : 1 covalent complex; D, C plus 5 nmol ferredoxin I; E, C plus 10 nmol ferredoxin I; F, C plus 15 nmol ferredoxin I; G, C plus 20 nmol ferredoxin I in a final vol. of 1.0 ml. The percentage of reduction by pyruvate dehydrogenase is obtained by comparison with the fully dithionite-reduced state.

blank with no electron acceptors shows that the $A_{418.2}$ increase is only due to the cytochrome reduction. Another blank, without pyruvate dehydrogenase-containing extract, shows that sodium pyruvate cannot reduce cytochrome c_3. The addition of sodium pyruvate to the reaction mixture containing the covalent complex as final electron acceptor induces a slight $A_{418.2}$ increase which corresponds to 5% of cytochrome c_3 reduction (Fig. 4c). Thus, pyruvate dehydrogenase cannot give its electrons to cross-linked cytochrome c_3. The low reduction is probably due to a heterogeneity of the covalent complex population. On the other hand, when free ferredoxin I is added to this reaction mixture in a ratio of 1,2,3 or 4 ferredoxin subunit(s) per covalent complex, there is an increase of the $A_{418.2}$ which corresponds to a final value of 86% of cytochrome c_3 reduction (Fig. 4D–G). Therefore, free ferredoxin I allows coupling between pyruvate dehydrogenase and cross-linked cytochrome c_3.

Reduction by hydrogenase

When pure hydrogenase is added to a mixture containing cytochrome c_3 alone as electron acceptor, under an H_2 atmosphere, there is an increase of A_{552} showing 89% of cytochrome c_3 reduction.

This experiment shows that pure hydrogenase can give its electrons directly to cytochrome c_3. The addition of ferredoxin I does not prevent this electron transfer. Blanks with hydrogenase alone without electron acceptors, or cytochrome c_3 alone without hydrogenase show that the A_{552} increase is only due to the cytochrome reduction and that hydrogen cannot directly reduce cytochrome c_3. The addition of pure hydrogenase to the reaction mixture containing the covalent complex as final electron acceptor induces an increase of A_{552} which corresponds to 89.5% of cytochrome c_3 reduction. This percentage shows that hydrogenase can give its electrons to cross-linked cytochrome c_3 as well as to free cytochrome c_3. Therefore, cross-linking with ferredoxin I does not prevent electron transfer from hydrogenase to cytochrome c_3.

Discussion

Studies of electron transfer proteins have allowed the determination of several structural parameters of the oxidoreduction proteins interaction [2–4]. Therefore, it can be postulated that the site of interaction between the physiological partners cytochrome c_3 ($M_r = 13000$) and ferredoxin I also includes a ring of positively charged residues around one heme edge of cytochrome c_3, but the fact that cytochrome c_3 is a tetrahemic protein raises a difficult problem in the identification of the hemes which are implicated in the interaction with the ferredoxin. ^{1}H-NMR experiments point out that cytochrome c_3 and ferredoxin I form a specific bimolecular complex in which one or two hemes are involved in the high-affinity interacting site [17].

To identify the heme(s) involved in the binding site, several approaches may be considered. One is computer fitting of the two structures, using a computer graphics procedure, which matched complementary charge regions on the two proteins. A model for this interaction, derived from the known structure of cytochrome c_3 and a predicted model of ferredoxin I, is in progress. The most convincing approach would be to co-crystallize the two proteins. Presently, we have chosen to determine the site of binding experimentally by chemical cross-linking. This approach offers the advantage of a stable derivative amenable to analysis. Among the several cross-linking agents generally used, carbodiimides seem to be the best suited cross-linkers for the study of the complex between proteins that are mainly stabilized by electrostatic interactions. Carbodiimides are expected to form amide bonds between carboxyl and amino groups that allow such interactions, without introduction of foreign spacer arms. The difficulty is that cross-linking might also occur outside of the native binding site. Thus, it is very important to demonstrate that the covalent complex is of a structure similar to that of the native non-covalent complex in order to exclude possible artefacts.

Several observations indicate that cross-linking promoted by CME-CDI is a specific process:
(1) The increase of the ionic strength which promotes the dissociation of the native non-covalent complex, prevents the cross-linking reaction between cytochrome c_3 and ferredoxin I.
(2) There is no covalent complex formation when another acidic protein (cytochrome c_3 ($M_r = 26000$)) is used as unspecific partner of cytochrome c_3 in the cross-linking reaction.
(3) Other studies have shown that the reaction of carbodiimide with clusters of carboxyl groups is favored [26,27] and these clusters are generally situated at the interacting site.
These observations suggest that the covalent reaction takes place after the formation of the native non covalent complex.

The cross-linked complex of $M_r = 24000$ may be compared to native complex throughout two properties: first, the stoichiometry of the association is one cytochrome c_3 molecule for one ferredoxin I subunit; second, the cross-linked cytochrome c_3 is fully reduced by dithionite and the spectrum does not show any perturbation due to the cross-linking reaction. Therefore, the covalent complex ($M_r = 24000$) seems to be a valid model of the native electron transfer complex.

In the covalent complex, the high-affinity binding site is blocked in the interaction with ferredoxin I. Therefore, in order to understand the role of the four hemes, it is interesting to assay the electron transfer activities in the two reactions described in (Fig. 1).

Ferredoxin I is necessary for the electron transfer to occur between pyruvate dehydrogenase and cytochrome c_3. Only 5% of cytochrome c_3 reduc-

tion in the 1 : 1 covalent complex is observed when pyruvate is added. This fact could be interpreted as a blocking of the ferredoxin interacting site and an inaccessibility of the (Fe-S) cluster which abolishes electron transfer from pyruvate dehydrogenase to ferredoxin. Thus, the interacting site of ferredoxin for pyruvate dehydrogenase and cytochrome c_3 might be the same. The 5% reduction of cross-linked cytochrome c_3 can be due to a heterogeneity of the population, in a small percentage of which cross-linked ferredoxin allows the electron transfer from pyruvate dehydrogenase to the cytochrome. However, the possibility of a non-functional covalent complex formation cannot be ruled out and could explain these results. But the specificity and validity of the covalent complex discussed above suggest that the first interpretation is the most probable. The addition of free ferredoxin to the covalent complex restores the reduction. Free ferredoxin I can take electron to pyruvate dehydrogenase and then reduces cross-linked cytochrome. In this case, one or several sites of lower affinity for ferredoxin could be postulated. On the other hand, there are no differences between reduction of free and cross-linked cytochrome c_3 by hydrogenase under an H_2 atmosphere. Two possible explanations can be offered for this: first, the cross-linked ferredoxin does not hinder the interaction between hydrogenase and cytochrome c_3, therefore, the electron transfer interaction domain for hydrogenase is different from the ferredoxin one. Second, the interacting site of high affinity for hydrogenase and ferredoxin is the same, but one or several other sites of lower affinity for hydrogenase could allow the electron transfer. To date, it is not possible to choose between these different possibilities. Cross-linking of cytochrome c_3 with hydrogenase could help to bring a satisfactory answer. Therefore, these electron tranfer activities suggest that the hemes, which are not blocked in the cross-linking, could be implicated in the interaction with other redox partners of the chain, assuming an intramolecular electron exchange. The specificity of each of the four hemes for various physiological partners would be an interpretation for the existence of a multihemic cytochrome.

The cross-linking site localization through enzymatic or chemical cleavage of the covalent complex and obtained peptides sequencing together with crystallization of the covalently linked proteins will be the main continuations of this study.

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