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On the interaction between cytochrome *f* and plastocyanin

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The interaction between cytochrome *f* and its electron acceptor plastocyanin (PC) was studied. To address the question of which specific regions and which of the positively charged residues of cytochrome *f* are important for the interaction with the negatively charged residues of PC we have used two different experimental approaches. Cytochrome *f* was proteolytically cleaved and fragments that could bind to a PC-affinity column were isolated. The smallest of these fragments was analysed to give information on the minimum structural requirement for binding to PC. By this procedure, we identified a peptide of approx. 11 kDa, containing the heme binding site, and having an N-terminal sequence identical to that of the mature cytochrome *f*. This finding suggests that the first 90 amino acids of cytochrome *f* contain at least some of the residues interacting with PC. The second approach involved modification of Arg residues of cytochrome *f* with the specific chemical modifier, hydroxyphenylglyoxal (HPG). Cytochrome *f* modification was performed in the absence of PC to enable identification of residues that are protected from modification when PC is bound to cytochrome *f*. Two peptides containing Arg residues which are modified in the absence of PC, but are not modified when PC is present, were isolated. Sequence analysis of these two peptides revealed that Arg residues no. 88 and 154 of cytochrome *f* are the residues that are protected from modification when cytochrome *f* is bound to PC, suggesting a role for these residues in the binding of cytochrome *f* to PC.

Introduction

The transfer of electrons from the cytochrome b_6f complex to Photosystem I (PS I) requires the soluble copper protein plastocyanin (PC). The immediate electron donor to PC is cytochrome *f*, and this reaction occurs at the lumenal side of the thylakoid membrane. The crystal structure of both reduced [1] and oxidized PC [2] have been determined, and structural features related to its function have been revealed. The copper is coordinated by two histidines: a methionine and a cysteine. The region around His 87 of PC interacts with PS I, enabling the reduction of P-700. A negatively charged patch around Tyr 83 of PC, at amino acids 59–61 and 42–45, is believed to be important for the binding of cytochrome *f*. Chemical modification of these residues has inhibited the rates of cytochrome *f* oxidation and PC reduction [3,4]. In a recent crosslinking study of PC to cytochrome *f* by a carbodiimide, two residues at this region of PC have been identified as covalently linked to cytochrome *f*: Asp 44 and Glu 60 (Morand and Krogmann, unpublished data).

Since the crystal structure of cytochrome *f* is not available, there is little information as to which specific residues on cytochrome *f* are interacting with PC. The primary structure of cytochrome *f* [5,6] (see Fig. 6) reveals several features pertinent to its interaction with PC. The lumen exposed portion of cytochrome *f* amounts to 87% of its length, and this portion contains highly conserved Arg and Lys residues, which might be possible candidates for interaction with the negatively charged patch of PC. However, since the tertiary structure of cytochrome *f* is not known, one needs to rely on indirect methods in identifying these positively charged residues. Morand and Krogmann (unpublished data) suggest that Lys 187 of cytochrome *f* crosslinks to Asp 44 of PC, but they have not identified the other cytochrome *f* amino acid that crosslinks to Glu 60 of PC. Moreover, it is possible that the binding of PC to cytochrome *f* involves more than two ionic interactions, since the negatively charged patch of PC is composed of seven acidic residues.

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Abbreviations: HPG, hydroxyphenylglyoxal; PAGE, polyacrylamide gel electrophoresis; PC, plastocyanin; PS I, Photosystem I; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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To address the question of which specific regions and which of the positively charged residues of cytochrome *f* are important for the interaction with the negatively charged residues of PC, we have used two different experimental approaches. We proteolytically cleaved cytochrome *f* and isolated fragments that could bind to a PC-affinity column. The smallest of these fragments was analysed to give information on the minimum structural requirement for binding to PC. The second approach involved modification of Arg residues of cytochrome *f* with the specific chemical modifier hydroxyphenylglyoxal (HPG) [7]. Cytochrome *f* modification was performed in the absence or presence of PC to enable identification of residues that are protected from modification when PC is bound to cytochrome *f*. We demonstrate that a cytochrome *f* peptide of 88 N-terminal amino acids is sufficient for interaction with PC, and that two specific Arg residues are protected from modification when cytochrome *f* is bound to PC. This suggests a role for these residues in the interaction of cytochrome *f* with PC.

Materials and Methods

Spinach cytochrome *f* was purchased from Sigma and resuspended in 5 mM Tris-HCl buffer (pH 7.6), containing 0.5% sodium cholate. Plastocyanin was prepared from spinach leaves by an unpublished method used in our laboratory, and was a gift from R.K. Chain.

PC-affinity chromatography. Purified PC was coupled to CNBr-activated Sepharose 4B (Sigma) as previously described [8]. The column was equilibrated with 5 mM sodium phosphate buffer (pH 6.0), and under these conditions could bind cytochrome *f*. Cytochrome *f* was released by washing the column with 50 mM sodium phosphate (pH 6.0). The same procedure was applied to trypsin-digested products of cytochrome *f* as well.

Trypsin digestion of cytochrome f. Cytochrome *f* was digested with either 5% or 1% trypsin (Cooper) for 1 h or overnight, respectively, at room temperature. The reaction was terminated by addition of 1 μl of 1 M HCl.

SDS-PAGE analysis. Trypsin-digested samples of cytochrome *f* were resolved using denaturing SDS-PAGE with a 10–20% resolving gel and a 4% stacking gel, in a Laemmli buffer system [9]. Peptides were visualized by either Coomassie Blue stain or heme stain [10].

HPG modification of cytochrome f. Cytochrome *f* (32 nmol) was incubated with HPG (Pierce) (6 μmol) overnight in the dark. When PC-protection experiments were carried out, PC (500 nmols) was preincubated with cytochrome *f* for 3 h at 4°C. To ensure interaction between cytochrome *f* and PC, the ionic strength of the reaction mixture was 5 mM. Following the incubation, the reaction mixture was passed over a Sephadex G-100 column to separate cytochrome *f* from PC and from unreacted HPG. The degree of modification was estimated spectrophotometrically at 340 nm, using the absorption coefficient of $18.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

Reverse phase HPLC purification of peptides. Trypsin-digestion products of HPG-modified cytochrome *f* were lyophilized and resuspended in 0.1% trifluoroacetic acid (TFA) (Pierce). The mixture was loaded on LiChrosorb RP-18 reverse phase column (5 μm, 4 × 250 mm) (LKB), equilibrated with 0.1% TFA and eluted with a gradient of 0–70% acetonitrile (Fisher) containing 0.1% TFA over 60 min. The eluent was monitored at $A_{310\,nm}$ to detect peptides containing HPG-modified arginine residues. In some cases, the fraction of interest was repurified on the same column using a gradient of 0–35% acetonitrile containing 0.1% TFA during 10 min, 35–49% acetonitrile in 30 min and 49–70% acetonitrile in 5 min.

Amino acid sequencing. Gel-resolved peptides or HPLC-purified peptides were sequenced using an automated gas-phase amino acid sequencer. In the case of gel-resolved peptide, peptides were transfered from the gel to 'Immobilon transfer membrane' (Millipore). The membrane was then rapidly stained with Coomassie Blue and destained and dried. The band of interest was cut off the membrane and further analyzed for its amino acid sequence. HPLC-purified peptides were lyophilized, resuspended in 50% acetonitrile containing 0.1% TFA and analyzed.

Results

Digestion of cytochrome *f* with trypsin under non-denaturing conditions yields a mixture of peptides of different lengths. When this mixture is fractionated on a PC-affinity column, some of these peptides bind to the column. Elution of the bound peptides with higher salt concentration (50 mM sodium phosphate buffer, pH 6.0) reveals four Coomassie Blue stainable peptides, ranging from 11–26 kDa in MW (Fig. 1 lane 1). Two of these peptides also stain for heme, indicating that these peptides, including the 11 kDa fragment, contain the heme binding site (Fig. 1 lane 2). Separation of the peptide mixture eluted from the PC-affinity column by reverse-phase HPLC reveals an elution profile with at least ten different fragments (Fig. 2 trace A). Monitoring the absorbance of the eluent at 420 nm (the Soret band of cytochrome *f* (Fig. 2 trace B) allows us the identification of heme-containing fragments. SDS-PAGE analysis of the fractions absorbing at 420 nm shows that the two main heme-stained peptides shown in Fig. 1 are separated from each other (Fig. 3 lanes 2 and 3; fraction 1 does not show any heme-stained band; the dark diffused spots at the bottom of the gel are free hemes dissociating from the peptides during electrophoresis). Further analysis of the fragment of molecular weight approx. 11 kDa was carried out in order to detail its exact composition. Either one of the two Arg residues on the N-terminal side of the heme-binding site

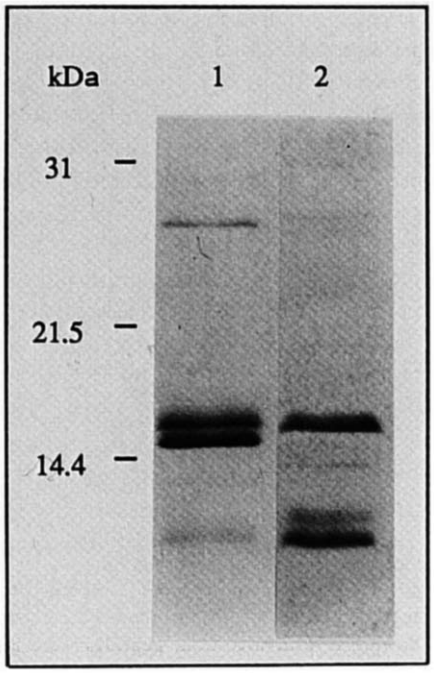

Fig. 1. Cytochrome *f* fragments binding to PC. Trypsin-digestion products of cytochrome *f* were fractionated on a PC-affinity column. Bound peptides were eluted from the column and resolved by SDS-PAGE. Lane 1: Coomassie Blue stained peptides; lane 2: heme-stained peptides.

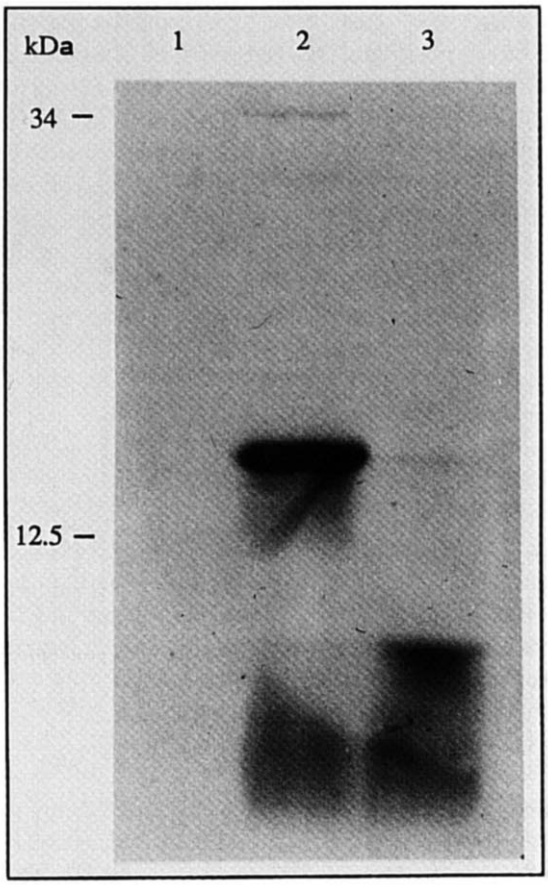

Fig. 3. Purification of heme-containing peptides. The three peaks absorbing at 420 nm (Fig. 2B) were analyzed by SDS-PAGE and stained for heme. Lanes 1–3 represents the contents of the peaks in Fig. 2.

(Arg 13 or Arg 18), or the N-terminal of the mature protein itself (see Fig. 6), might be the N-terminal of the 11 kDa fragment. Microsequencing of this fragment shows a sequence of Tyr-Pro-Ile-Phe-Ala, identical to the N-terminal of the mature cytochrome *f* (see Fig. 6). The N-terminal sequence information, together with the estimated size of this fragment, based on its migration under SDS-PAGE conditions, suggests that the first 90 amino acids of cytochrome *f* contain at least some of the positively charged residues involved in the interaction with PC.

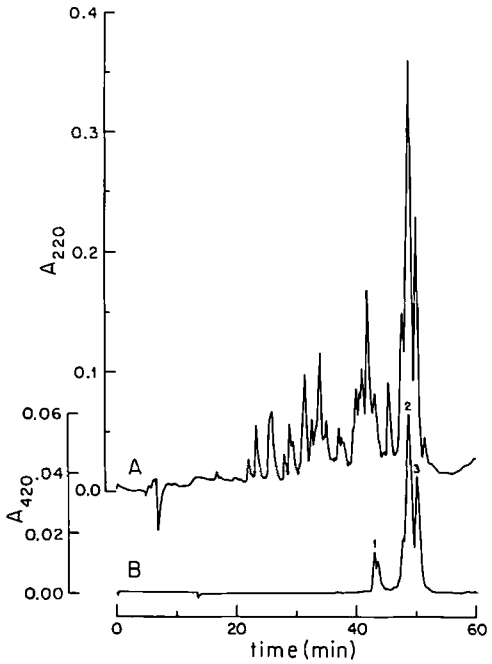

Fig. 2. Reverse-phase HPLC separation of cytochrome *f* fragments binding to PC. Trypsin-digestion products of cytochrome *f* binding to PC affinity column were resolved on reverse-phase HPLC column and eluted as described in Materials and Methods. Elution was monitored at 220 nm (A) or 420 nm (B).

The 11 kDa fragment binding to PC possess six conserved arginine and lysine residues which might be involved in the interaction with PC. In order to identify specific residues interacting with PC in this region, as well as in other regions, of the cytochrome *f* molecule, we used an alternative approach. Using the specific arginine chemical modifier HPG, we modified cytochrome *f* in the presence and absence of PC. HPG has a maximal absorption at pHs above neutral at 335 nm. At acidic pH values its peak shifts toward shorter wavelengths, with a maximum at 285 nm. Comparison of the absorption spectra of HPG-modified and non-modified cytochrome *f* (Fig. 4) shows higher absorbance values of the modified protein at the wavelength region of 300 to 320 nm, merging with the 280 nm peak of the protein. The maximal absorbance dif-

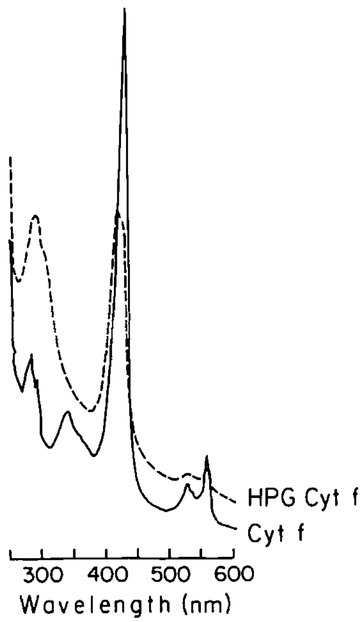

Fig. 4. Absorption spectra of HPG-modified and non-modified cytochrome *f*. Cytochrome *f* was modified as described in Materials and Methods. Optical absorption of native (———) and HPG-modified (- - - - - -) cytochrome *f* was measured. The absorbance was recorded on different scales: 0.2 and 1.0 full scale for the native and the modified forms, respectively.

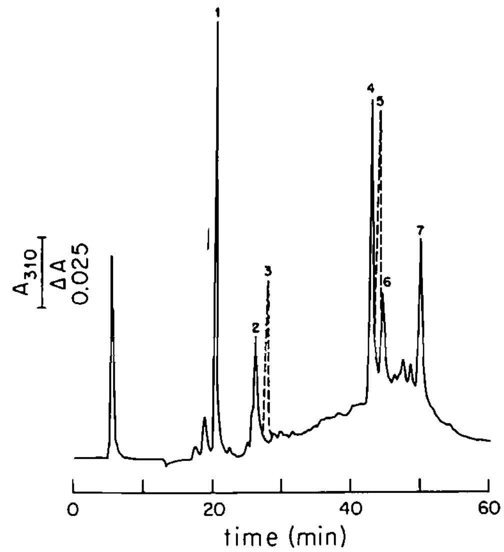

Fig. 5. Reverse-phase HPLC chromatography of trypsin-digested HPG-modified cytochrome *f*. Cytochrome *f* was modified with HPG in the presence or absence of PC as described in Materials and Methods. Following trypsin digestion, peptides were separated and the elution was monitored at 310 nm to detect peptides containing modified Arg residues. The elution profile of peptides of cytochrome *f* modified in the presence of PC is presented (———). When the modification was performed in the absence of PC, two additional peaks were observed (- - - - - -).

10 20
Tyr Pro Ile Phe Ala Gln Gln Gly Tyr Glu Asn Pro Arg Glu Ala Thr Gly Arg Ile Val

A 30 40
Cys Ala Asn Cys His Leu Ala Asn Lys Pro Val Asp Ile Glu Val Pro Gln Ala Val Leu

50 ---------------------------> 60
Pro Asp Thr Val Phe Glu Ala Val Val Arg Ile Pro Tyr Asp Met Gln Leu Lys Gln Val

70 80
Val Leu Ala Asn Gly Lys Lys Gly Gly Leu Asn Val Gly Ala Val Leu Ile Pro Glu Gly
5

90 ------------------100
Phe Glu Leu Ala Pro Pro Asp Arg Ile Ser Pro Glu Met Lys Glu Lys Met Gly Asn Leu
5

---> 110 120
Ser Phe Gln Ser Tyr Arg Pro Asn Lys Gln Asn Ile Leu Val Ile Gly Pro Val Pro Glu

130 140
Gln Lys Tyr Ser Glu Ile Thr Phe Pro Ile Leu Ala Pro Asp Pro Ala Thr Lys Lys Asp

----------------------> 150 160
Val His Phe Leu Lys Tyr Pro Ile Tyr Val Gly Gly Asn Arg Gly Arg Gly Gln Ile Tyr
3

170 180
Pro Asp Gly Ser Lys Ser Asn Asn Thr Val Tyr Asn Ser Thr Ala Thr Gly Ile Val Lys

------------190---------> 200
Lys Ile Val Arg Lys Glu Lys Gly Gly Tyr Glu Ile Asn Ile Ala Asp Ala Ser Asp Gly

210 220
Arg Glu Val Val Asp Ile Ile Pro Arg Gly Pro Glu Leu Leu Val Ser Glu Gly Glu Ser

230 240
Ile Lys Leu Asp Gln Pro Leu Thr Ser Asn Pro Asn Val Gly Gly Phe Gly Gln Gly Asp

250 B 260
Ala Glu Val Val Leu Gln Asp Pro Leu Arg Ile Gln Gly Leu Leu Phe Phe Phe Ala Ser

B 270 280
Val Ile Leu Ala Gln Ile Phe Leu Val Leu Lys Lys Lys Gln Phe Glu Lys Val Gln Leu

Ser Glu Met Asn Phe

Fig. 6. Amino acid sequences of cytochrome *f*. Amino acid sequence of spinach cytochrome *f* [5]. Box A: the heme-binding site; box B: the membrane spanning helix; - - - - - -, N-terminal sequence of peptides included in peak no. 3, Fig. 5; ———, purified peptides of peaks no 3 and 5 of Fig. 5, containing modified arginine residues.

ference is observed around 310 nm. This property of the modified protein allowed us to detect fragments containing modified arginine residues. Using the absorption coefficient of $18.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [7], we could also estimate the number of arginine residues of cytochrome *f* that are modified; when the protein is in its native form, four arginine residues are modified by HPG (4 mol modified Arg : mol cytochrome *f*).

Arginine residues of cytochrome *f* which are not involved in intra-molecular ionic interactions are likely to get modified by HPG. If any of these residues are also capable of interacting with PC, these would be protected from modification when PC is present during the modification reaction. Based on this assumption, we preincubated reduced cytochrome *f* with or without oxidized PC under low ionic strength conditions, to allow binding of cytochrome *f* to PC, HPG was then added to both samples in order to modify available arginine residues. During this incubation PC was reduced, indicating that it was interacting with cytochrome *f*. Following modification, the modified cytochrome *f* was separated from excess HPG and from PC by gel filtration, and digested with trypsin. The peptide mixture was fractionated by reverse-phase HPLC chromatography, and the absorbance of the eluent was monitored at 310 nm to detect peptides containing modified arginines. A typical elution profile of such an experiment is presented in Fig. 5. Peak no.1 represents free HPG released from the modified peptides during chromatography, due to low pH conditions (the modification reaction is reversible in acidic conditions [7]). Peaks no. 2–7 represent peptides containing modified arginine residues. From these results it is clear that peaks no. 3 and 5 are missing when the modification is carried out in the presence of PC. These results indicate that these peptides contain arginine residues which are protected from modification when PC is bound to cytochrome *f*, presumably due to their involvement in the interaction with PC. It should be noted that the differential modification was dependent on pre-incubation of cytochrome *f* with PC prior to the incubation with HPG, and high ratio of PC to cytochrome *f* (15 : 1).

Amino acid sequence analysis of the first five amino acids of peak 3 revealed that it was composed of four different peptides, starting at Ile-51, Met-97, Tyr-146 and Gly-188 (see Fig. 6). These peptides were repurified using different elution conditions (see Materials and Methods), and the fraction with highest absorbance at 310 nm was sequenced again. The sequence of the entire peptide spans from Tyr 146 to Arg 154, with this arginine residue being the only arginine in the peptide. Peak no. 5 was also sequenced, and was found to match the stretch from Gly-67 to Arg-88. Taken together, it seems that arginine residues no. 88 and 154 of cytochrome *f* are modified by HPG when PC is absent from the reaction mixture and are protected from modification when PC is bound to cytochrome *f*. This observation suggest a role for these residues in the binding of cytochrome *f* to PC.

Discussion

Electron transfer between cytochrome *f* and PC in the electron transport from the cyt b_6f complex to PS I is well established. Detailed structural features of PC, derived from its elucidated three-dimensional structure [1,2], together with chemical modification experiments [3], led to the identification of specific residues on PC believed to be involved in the interaction with cytochrome *f*. However, no information on the corresponding regions and specific residues of cytochrome *f* involved in this interaction is available. We therefore used two different approaches in an attempt to identify amino acids in cytochrome *f* interacting with PC.

Cleavage of cytochrome *f* with trypsin under non-denaturing conditions yield several peptides of different lengths. Some of these peptides can bind to PC (Fig. 1) based on a PC-affinity column procedure. We separated cytochrome *f* fragments that can bind to a PC-affinity column and analyzed the amino acid sequence of the shortest of these peptides, assuming that this peptide contains at least some of the positively charged residues interacting with PC. This assumption would hold of course only if the cleavage products retain the same conformation they have as a part of the native cytochrome *f* molecule. Using this approach, we identified a peptide about 11 kDa, containing the heme binding site (Fig. 1), and having an N-terminal sequence identical to that of the mature cytochrome *f*. This finding suggests that the first 90 amino acids of cytochrome *f* contain at least some of the residues interacting with PC. Eight highly conserved Lys and Arg residues are located in this region of the cytochrome *f* molecule, and they have the potential to bind the acidic patch of PC. Since the identified fragment contains also the heme-binding site, interaction between this region of cytochrome *f* and PC could bring the heme donor close to the copper acceptor. This notion does not infer that residues from other regions of the cytochrome *f* molecule cannot be involved in the interaction with PC.

In an attempt to look at specific residues involved in the interaction, we tried to modify basic residues of cytochrome *f* in the presence and absence of PC. We assume that residues which are not involved in ionic interactions within the protein will be available for modification. Identification of residues that can be modified in the absence of PC, but are protected from modification when PC is bound to cytochrome *f* could suggest a role for these residues in the interaction between the two molecules. Unfortunately, we could use this approach only for arginine residues, since there is no available specific chromophoric chemical modifier

for lysine. Using the Arg-specific chemical modifier, HPG, we could modify cytochrome *f*. Based on the spectral properties of the modified protein, four out of the total of 11 Arg residues were modified. The modified protein was then digested with trypsin, and peptides containing modified arginine residues were separated. Two such peptides were absent when the modification was performed on cytochrome *f* in the presence PC (Fig. 5). Sequence analysis of these two peptides revealed that Arg residues no. 88 and 154 are the residues that are protected from modification when cytochrome *f* is bound to PC, suggesting a role for these residues in the binding of cytochrome *f* to PC.

Arg-88 falls within the region of the first 90 amino acids that was identified in the first part of this study as the smallest fragment of cytochrome *f* that can bind to PC, and thus its role in the interaction is supported. Arg-154 is located well beyond this region, and its role cannot be supported by the binding experiments. Still, it is possible that the folding of the cytochrome *f* molecule brings these two distinct Arg residues together to such a conformation which allows both of them to interact with the negatively charged patch of PC.

The suggested binding site for cytochrome *f* on the PC molecule is composed of seven acidic residues. Theoretically, they all can be involved in the interaction of the two proteins. Therefore, it is possible that other basic residues are involved in that interaction. It should be noted that our attempt to identify specific residues by modification of exposed and protected sites was limited to Arg residues. Cytochrome *f* has many conserved Lys residues that might be involved in the interaction as well. Support for this possibility comes from crosslinking experiments in which Lys-187 of cytochrome *f* was identified as crosslinked to Asp-44 of PC, together with an unidentified residue that crosslinks to Glu-60 of PC (Morand and Krogmann, unpublished data). However, cross-linking is not necessarily indicative of a binding interaction.

The results of this work, although indirect, suggest that Arg residues no. 88 and 154 of cytochrome *f* are involved in the binding of cytochrome *f* to its electron acceptor PC. This does not rule out the possibility that other basic residues, especially lysines, are involved in the interaction. However, ultimate identification of the involved residues will obviously come from the elucidation of the tertiary structure of cytochrome *f*, together with activity assays of site-directed mutant cytochrome *f* molecules in which these specific amino acids have been altered.

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