Biochimica et Biophysica Acta 848 (1986) 324–332
Elsevier

BBA 41930

Nearest-neighbor relationships of the constituent polypeptides in plastoquinol-plastocyanin oxidoreductase

Eric Lam *

Division of Molecular Plant Biology, 313 Hilgard Hall, University of California, Berkeley, CA 94720 (U.S.A.)

(Received June 17th, 1985)

Key words: Cross-linking; Glutaraldehyde; Plastoquinol-plastocyanin oxidoreductase; Cytochrome b_6/f complex; (Spinach chloroplast)

The nearest-neighbor relationship among the constituent polypeptides of the isolated plastoquinol-plastocyanin oxidoreductase from spinach chloroplasts has been investigated. (1) The isolated plastoquinol-plastocyanin oxidoreductase (the b_6/f complex) is treated with various concentrations of the cross-linker glutaraldehyde. The treated b_6/f complexes are then analyzed by SDS-polyacrylamide gel electrophoresis coupled with the immunodecoration of cross-link products by specific antibodies for each of the four prominent constituent polypeptides. Cytochrome b_6 is found to be most resistant to forming any intermolecular cross-link products. At low concentrations of glutaraldehyde, the 'Rieske' iron-sulfur (Fe-S) protein and subunit IV of the b_6/f complex, however, appear to form cross-link products with a relative molecular weight of 35000. Dimers of cytochrome f and cytochrome f/Rieske protein cross-link products can also be detected. (2) When a Rieske Fe-S protein-depleted b_6/f complex is used in place of the control b_6/f complex, cytochrome b_6 is less resistant to intermolecular cross-linking, while subunit IV does not form any 35 kDa cross-link product, unlike the case in control b_6/f complex. Subunit IV is concluded to be closely associated with the Rieske Fe-S protein. This provides evidence that subunit IV is a bona fide component of the cytochrome b_6/f complex, although no function can yet be assigned to it. The results are discussed in relationship to the spatial and functional relationships among the components of the b_6/f complex.

Introduction

The cytochrome b_6/f complex of the thylakoid membrane functions to connect the two photosystems of the photosynthetic electron-transport chain. It has many functional, as well as structural similarities with the ubiquinol-cytochrome c oxidoreductases of mitochondria and photosynthetic bacteria [1]. The isolated b_6/f complex is composed of four polypeptides with apparent molecular masses of 33, 23, 20 and 17 kDa [2]. The stoichiometry of these four polypeptides has been reported to be 1 : 1 : 1 : 1 based on staining intensities of the different peptides after their separation by polyacrylamide gel electrophoresis [3]. The 33 kDa peptide runs as a doublet at times and is demonstrated to be cytochrome f, while the 23 kDa polypeptide was shown to be cytochrome b_6 [3]. The 20 kDa polypeptide is identified to be the Rieske Fe-S protein [4]. Recently, Hauska et al. [5] concluded that plastocyanin binds directly to cytochrome f from chemical cross-linking studies. The

* Present address: Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A.

Abbreviations: Cytochrome b_6/f complex, plastoquinol-plastocyanin oxidoreductase; $(b_6/f)_{RD}$ complex, Rieske Fe-S protein-depleted cytochrome b_6/f complex; duroquinone, tetramethylquinone; Mes, 4-morpholineethanesulfonic acid.

function of the 17 kDa polypeptide, subunit IV, is still unknown. In addition to these four prominent polypeptides the isolated b_6/f complex contains a number of lower molecular mass (less than 15 kDa) peptides. These peptides have been suggested to be part of the intact b_6/f complex, since they have been observed in all the isolated quinol-cytochrome *c* type oxidoreductases [3,5]. The peptide at about 14 kDa might be a degradation product, while the smallest but more prominent band(s) under 10 kDa seems to co-purify with the b_6/f complex [3]. From a sequence comparative study, Widger et al. [6] suggested that the 17 kDa subunit in the b_6/f complex contains the functions of the COOH-terminal end of the mitochondrial cytochrome *b* polypeptide and that the single cytochrome *b* gene of mitochondria is equivalent to the genes of cytochrome b_6 and subunit IV of chloroplasts.

In the present study, the nearest neighbor relationship among the polypeptides of the b_6/f complex has been investigated. This has been addressed for mitochondrial Complex III (reviewed in Ref. 7). Similar studies have been initiated for Photosystem I reaction center preparation by Cox and Miller [8]. These studies have utilized cleavable cross-linkers in conjunction with two-dimensional gel electrophoresis to analyze cross-link products. In the present investigation, a different approach is taken where a covalent cross-linker is used to produce stable products which are then analyzed by immuno-blotting techniques with specific antibodies for each of the four main polypeptides in the b_6/f complex. The approach affords higher sensitivities and does not have the streaking problem often encountered with two-dimensional gels. Also, any covalent cross-linker can be used and thus we are not restricted to specific functional groups that one can modify. In this study, a relatively non-specific cross-linker, glutaraldehyde, is chosen as the modifier. Its short length, about 0.7 nm, restricts the formation of cross-link products to only closely situated polypeptides under low reactant concentrations where random collisions are minimized kinetically. The results indicate a close relationship between the Rieske Fe-S protein and subunit IV. Evidence for the formation of cytochrome f/Rieske protein cross-link product and cytochrome f dimers is also observed.

Experimental procedures

Materials

Plastoquinol-plastocyanin oxidoreductase is purified from greenhouse-grown spinach by the procedure of Hurt and Hauska [2] with the modification that octylglucoside and sodium chlolate replaced Triton X-100 in the sucrose gradient step [9]. The Rieske Fe-S protein is extracted by the procedure of Hurt, E. et al. [4], using hydroxyapatite column chromatography. The Rieske Fe-S protein is eluted by 0.5% Triton X-100 and 20 mM potassium phosphate (pH 7.2). Subsequently, the depleted complex is washed off by 0.05% Triton X-100 and 200 mM potassium phosphate, pH 7.2.

Octylglucoside, sodium cholate, Nonidet P40, Triton X-100, and glutaraldehyde are obtained from Sigma Chemical Company. All other reagents are of the highest grade available. Antibodies for spinach cytochrome f were a kind gift of Wilma and Amy Klecan. Maize Rieske Fe-S protein, cytochrome b_6 and subunit IV antisera are obtained from Dr. Alice Barkan (Berkeley, CA). Duroquinol is obtained from K and K Chemical Company. Plastocyanin is obtained from R.K. Chain.

Methods

Cross-linking of plastoquinol-plastocyanin oxidoreductase

The b_6/f complex is dialyzed overnight against 2 l 100 mM potassium phosphate (pH 7.6)/0.05% Triton X-100 at 4°C. The dialyzed complex (15–25 μM cytochrome f) is then reacted with various concentrations of glutaraldehyde at 22°C for 5 min. Glutaraldehyde is diluted from a 1% fresh stock solution. Tris-HCl (pH 7.5) from a 2 M stock solution is then added to a final concentration of 50 mM in order to quench the reaction. The mixture is then allowed to incubate at room temperature for 30 min before use.

SDS-polyacrylamide gel electrophoresis and immuno-blot analysis

For SDS-polyacrylamide gel electrophoresis analysis, the cross-linked b_6/f complex is used immediately after its preparation without further

storage. To the sample, about 200 μl, are added 300 μl water, 600 μl solubilization buffer and 20 μl PMSF (100 mM stock solution). The solubilization buffer contains 8 M urea/10% glycerol/5%SDS/10% β-mercaptoethanol/0.005% bromophenol blue. The mixture is incubated at room temperature for at least 3 h before use and is stored in this form at −80°C for future SDS-polyacrylamide gel electrophoresis analysis. The gel system employed is that of Chua [10]. An 8–16% linear acrylamide gradient is employed for the separation gel. The stacking gel is 3% in acrylamide. The gel is run at 6–8 mA for about 15 h. The resultant gel is either stained with Coomassie blue to visualize the polypeptides or used for immuno-blot analysis as described previously [11]. The separated polypeptides are transferred to CNBr-treated filter paper by a Trans-Blot apparatus (BioRad). A steady current of 1.5 A for 4 h is used for the electrotransfer. The filter paper is then washed with 1% bovine serum albumin, 1 M glycine (2 × 30 min) and then subsequently incubated with the antibodies for the peptide in question overnight. The blot is then washed with a solution containing 0.15 M NaCl/0.1% Nonidet P40/0.1% bovine serum albumin/10 mM sodium phosphate (pH 7.3). The antibody is then visualized by ^{125}I-labeled protein A (Amersham) blotting.

The densitometric scans of the Coomassie blue-stained gel lanes are obtained with a modified Gilford spectrophotometer connected to a Hewlett-Packard integraor/plotter. The optical density at 580 nm is followed. The area under the various peaks are obtained electronically from the integrating routine. Control studies using various amounts of the isolated cytochrome b_6/f complex demonstrates that this method is valid for the concentration range of our study (data not shown).

Activity and concentrations of the cytochrome b_6/f complex

The activity of the b_6/f complex is determined by the rate of plastocyanin reduction using duroquinol as reductant. A stirred cuvette (mixing time, up to 1 s) is used. The reduction of plastocyanin is followed by an Aminco DW2a spectrophotometer operating in the dual-beam mode. The reference wavelength is 500 nm, while the sample wavelength is 580 nm. The reaction mixture (2.5 ml) contains 0.84 nmol cytochrome f, 26 nmol plastocyanin (90% oxidized) in 20 mM Mes (pH 6.0). 10 μl of duroquinol (2 mg/ml) is then added to start the reaction. The rate of plastocyanin reduction in the absence of cytochrome b_6/f complex by duroquinol is subtracted as the uncatalyzed rate. All activities are measured in room temperature.

The concentration of cytochrome f is determined spectrophotometrically by reduced-minus-oxidized spectrum. Ascorbate and ferricyanide are used as the reductant and oxidant, respectively. The spectrum is obtained on a Cary 219 spectrophotometer equipped with automatic baseline correction. An extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ is used for the alpha band of the reduced cytochrome.

Results

Cross-linking of the isolated b_6/f complex

The isolated b_6/f complex is modified with various concentrations of glutaraldehyde at pH 7.8 in a potassium phosphate medium. The cross-linked b_6/f complexes are then analyzed by SDS-polyacrylamide gel electrophoresis after solubilization in urea and SDS. Under our conditions, cytochrome f runs as a band at 35 kDa, while cytochrome b_6, the Rieske Fe-S protein and subunit IV have apparent molecular weights of 20, 18 and 16 kDa, respectively. A new band of low staining intensity can be detected at an apparent molecular weight of about 36 kDa when higher glutaraldehyde concentrations are used (i.e., 0.04% or more). No other new bands are apparent although the amount of subunit IV and the Rieske protein appears to decrease significantly even at the lowest concentration of glutaraldehyde used. Densitometric traces of the stained-gel lanes are then analyzed in order to quantitate the covalent cross-linking of the various constituent peptides. The resultant data is plotted in Fig. 1A. It is evident that cytochrome b_6 is the most resistant toward cross-linking with other polypeptides. The Rieske Fe-S protein and subunit IV disappear from their original migration position in a biphasic manner. The close correlation of their disappearance suggests the formation of a cross-link

product between these two polypeptides. The disappearance of cytochrome *f* is not directly correlated with the other polypeptides in the complex.

The effects of glutaraldehyde treatment on the activity of the isolated b_6/f complex are also examined. As shown in Fig. 1B, the activity of the plastoquinol-plastocyanin function is hardly affected even up to 0.04% glutaraldehyde, where about 70% of the Rieske Fe-S protein and subunit IV has been cross-linked. In fact, the effect of

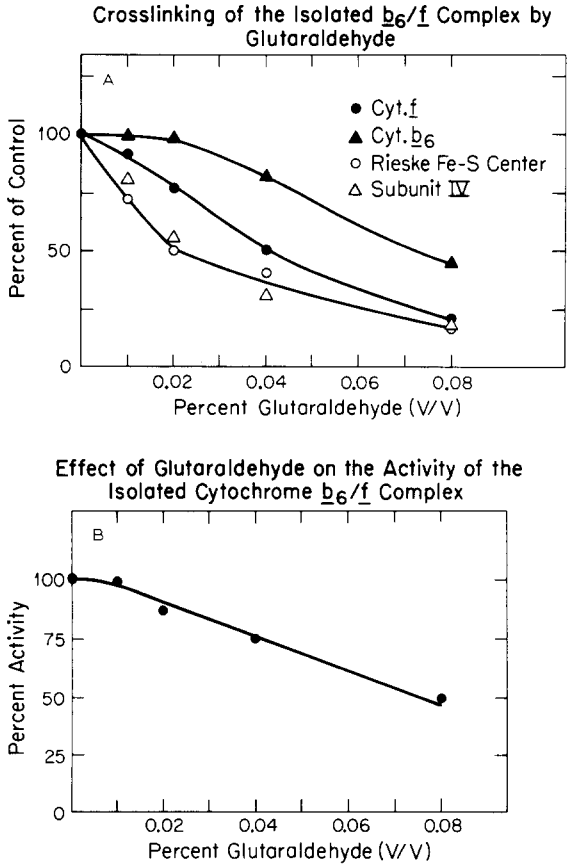
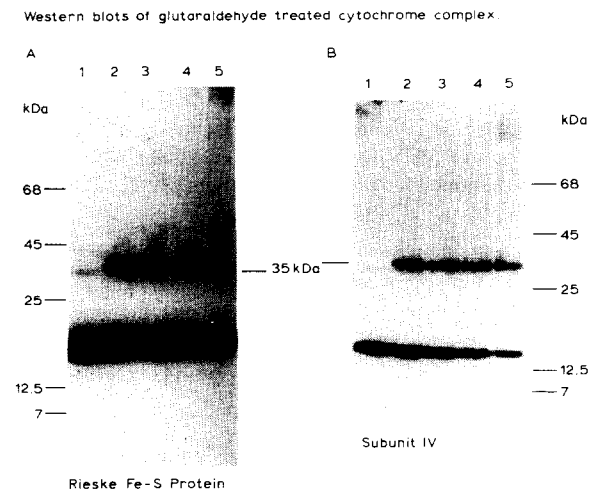


Fig. 1. Effects of glutaraldehyde treatment on isolated b_6/f complex. (A) After modification with glutaraldehyde, the isolated b_6/f complex is solubilized and then electrophoresed on an 8–16% linear acrylamide gradient gel as described in the experimental section. The polypeptides are stained with Coomassie blue and the lanes are then scanned for absorbance at 580 nm and their relative absorbance peak area plotted as percent of the control complex for each of the four prominent polypeptides. (B) The samples used for SDS-polyacrylamide gel electrophoresis analysis are used for plastoquinol-plastocyanin oxidoreductase activity after the treatment with glutaraldehyde. The assay conditions are described in the experimental section.

glutaraldehyde on the oxidoreductase activity seems to match the cross-linking of cytochrome b_6 (see Fig. 1A). This might suggest that modification of cytochrome b_6 by glutaraldehyde is the cause for the observed inhibition. For certain, however, we can conclude that cross-linking of the Rieske Fe-S protein to subunit IV does not directly affect the plastoquinol-plastocyanin oxidoreductase activity of the b_6/f complex.

Immuno-decoration of the SDS-polyacrylamide gel electrophoresis separated polypeptides is used to identify cross-linked products. Fig. 2A shows the fluorographs from immunoblots of the cross-linked b_6/f complexes with antibodies against the Rieske Fe-S protein and subunit IV. A new band at 35 kDa apparent molecular mass is evident in both cases. Similar results are obtained with gels which contained 2 M urea where this cross-link product is well separated from the cytochrome *f* band (data not shown). Thus, the 35 kDa band observed in gels stained for protein is positively identified as a product of the Rieske Fe-S protein and subunit IV. The combined molecular mass of 34 kDa for these two proteins in our gel system is in good agreement with the identification of this new band as a one-to-one cross-link product from the Rieske Fe-S protein and subunit IV. However, the possibility that dimers of these two polypeptides might fortuitously run at identical position in our gel systems cannot be ruled out completely.

The cross-linking of subunit IV has also been examined using the fluorograph of the immuno-blot probed with antibodies specific for subunit IV. The lanes in the immuno-blot from Fig. 2A are scanned for absorbance at 580 nm. Both the peak area under the 16 kDa (non-modified form) and 35 kDa (cross-linked form) bands are quantitated. Since the total amount of subunit IV is constant for each sample, the peak area for the control b_6/f complex is taken as 100% and the effects of glutaraldehyde cross-linking on the formation of the 35 kDa cross-link product and the 16 kDa non-modified form of subunit IV is shown in Fig. 2B. The disappearance of subunit IV from its normal migration position as quantitated by fluorograph scanning is compared with that of Coomassie-blue staining. It is evident that the two methods give identical results quantitatively. This



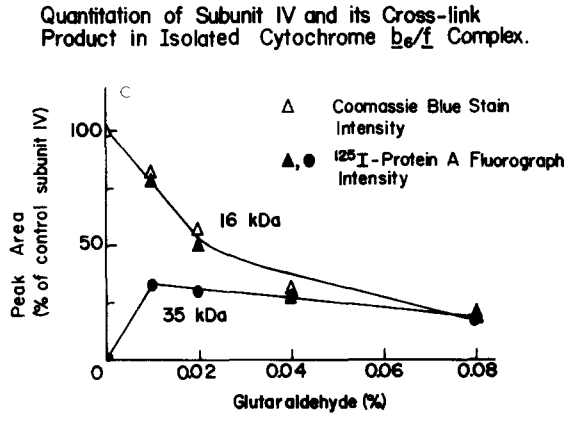


Fig. 2. Immuno-blots of glutaraldehyde-treated b_6/f complex. (A and B) The cytochrome b_6/f complexes used in Fig. 1 are probed with antibodies against the Rieske Fe-S protein and subunit IV separately. The conditions are identical to those of Fig. 1. (A) Antibodies against the Rieske Fe-S protein; (B), antibodies against subunit IV. It is observed that the Rieske Fe-S protein antibodies also has some cross-reactivity toward cytochrome b_6 and subunit IV. This prevented us from quantitating the exact amount of cross-link product at 35 kDa relative to that of the Rieske Fe-S protein in the control complex. However, the relative amount of radioactivity at this band as compared to the position at non-modified subunit IV between the two panels enables us to conclude that the antibodies do correctly identify a cross-link product involving the Rieske Fe-S protein. (C) The densitometric scans at 580 nm of the lanes in the fluorograph shown in part B are analyzed. The peak area for the control complex is taken as 100%. Also shown for comparison are the data in Fig. 1 for subunit IV.

observation demonstrates that under these conditions, the peak area under the bands in the fluorograph of the subunit IV immuno-blot is directly proportional to the amount of subunit IV. Quantitation of the 35 kDa cross-link product using this approach shows that in low concentration of glutaraldehyde, 30% of the total amount of subunit IV is converted into the 35 kDa product. At higher concentration of glutaraldehyde (i.e., 0.02% or more), the amount of the 35 kDa product did nto increase further although the amount of unmodified subunit IV continue to decrease. In fact, the amount of the 35 kDa product even dropped from about 30% to 22% of the total subunit IV with higher concentration of glutaraldehyde. This is most likely due to the formation of insoluble cross-link products with the other subunits in the cytochrome b_6/f complex which are not resolved in our gel electrophoresis system. Indeed, cross-reactive materials to subunit IV and other cytochrome complex antibodies at the top of the gel can be observed in the immuno-blots with longer exposure times (data not shown).

With prolonged incubation of glutaraldehyde, another band at about 53 kDa can also be observed to cross-react with the Rieske Fe-S protein antibody (data not shown). As shown in an immuno-blot analysis of the cross-linked complexes with cytochrome-*f*-specific antibodies (Fig. 3), the 53 kDa band is most likely a cross-link product between the Rieske Fe-S protein and cytochrome *f*. The higher titer of the cytochrome *f* antibody preparation is probably the reason for our detection of this product under the same conditions that we used for the blot of Fig. 2. Low yield of this cytochrome *f*/Rieske protein cross-link product as compared to that of the Rieske/subunit IV product suggests that the Rieske Fe-S protein is more closely situated with subunit IV than cytochrome *f*. A new form of cytochrome *f* which migrates with a relative molecular weight of about 36 000 can be detected also. At present, it is suggested that this represents intramolecularly cross-linked cytochrome *f*. The most prominent new cross-link product in the cytochrome *f* immuno-blot is the new band at about 70 kDa. Since antibodies against the other three b_6/f complex polypeptides do not cross react with peptides at this range (see figures 2 and 3), it is concluded that this band represents a dimeric form of cytochrome *f*. The cytochrome b_6 immuno-blot of the cross-linked complexes is shown in Fig. 3 also. As

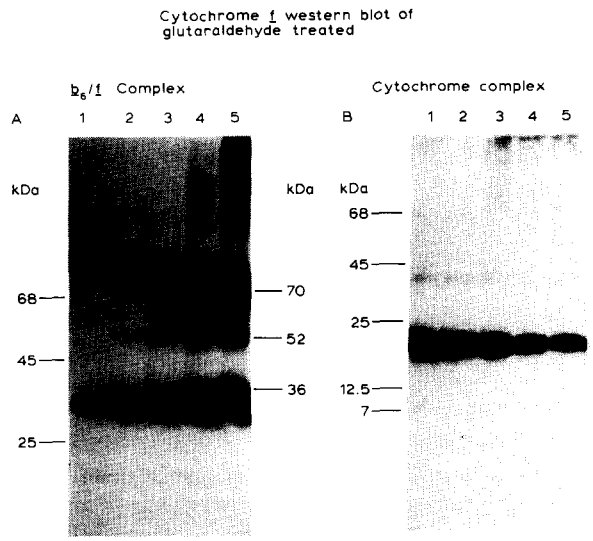


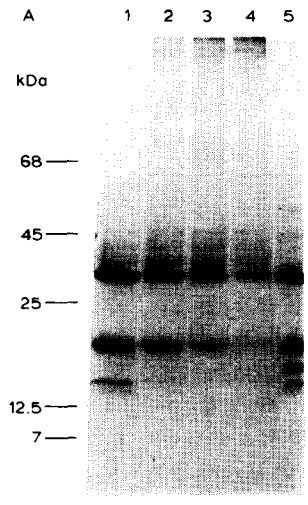
Fig. 3. Cytochrome f and b_6 immuno-blot of glutaraldehyde treated b_6/f complex. Same conditions are used as in Fig. 2. Panel (A), spinach cytochrome f antisera are used. Panel (B), maize cytochrome b_6 antisera are used.

expected from the densitometric analysis of the stained gel, a slow disappearance of the protein is observed. However, no discrete cross-link products can be identified. At higher concentrations of glutaraldehyde, highly cross-linked products which cross-react with the antibody are detected at the top of the gel.

Glutaraldehyde modification of Rieske-depleted b_6/f complex

The nearest-neighbor relationship in a b_6/f complex depleted of the Rieske Fe-S protein has also been studied. The reason for this investigation is two-fold. (1) To test the conclusions that subunit IV and cytochrome f form cross-link products with this protein after modification with glutaraldehyde. (2) To investigate whether other cross-link products can be detected as a result of the extraction of the Rieske Fe-S protein.

The analysis of the cross-linked complex by SDS-polyacrylamide gel electrophoresis is shown in Fig. 4. It is observed that cytochrome b_6 became more accessible to cross-linking by glutaraldehyde, and it is evident that the disappearance of cytochrome f from its normal position in the gel closely matches that of cytochrome b_6. Thus, it is possible that in the $(b_6/f)_{RD}$ complex, cytochrome



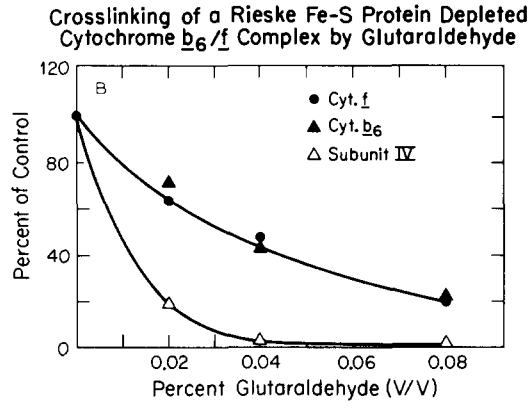


Fig. 4. Effects of glutaraldehyde treatment on a Rieske Fe-S protein-depleted cytochrome complex. (A) The $(b_6/f)_{RD}$ complex is used for cross-linking study in place of control b_6/f complex. Other conditions similar to that of Fig. 1. Lane 1, incubated with 50 mM Tris-HCl only; lane 2, treated with 0.02% glutaraldehyde; lane 3, treated with 0.04% glutaraldehyde; lane 4, treated with 0.08% glutaraldehyde; lane 5, untreated control b_6/f complex. (B) Densitometric analysis of the lanes in part A. The area under the peaks of interest in the scans of the various gel lanes are graphed as the percentage of the control $(b_6/f)_{RD}$ complex.

b_6/f cross-link products might be expected. The disappearance of subunit IV from its original position is practically completed at about 0.04% glutaraldehyde. This is substantially faster than in the case of intact b_6/f complex. Cytochrome f also appears to be slightly more susceptible to glutaraldehyde modification in the $(b_6/f)_{RD}$ complex. Immuno-blot of the glutaraldehyde-treated

$(b_6/f)_{RD}$ complexes with cytochrome b_6 antibodies shows essentially the same pattern as with intact b_6/f complexes (data not shown). No discrete cross-link product of cytochrome b_6 with cytochrome f can be observed. Only heavily cross-linked materials with high molecular masses are observed. The immuno-blots of $(b_6/f)_{RD}$ complex with cytochrome f and subunit IV antibodies are shown in Fig. 5. It is interesting to point out that the prominent band at 35 kDa observed in the subunit IV immuno-blot of the intact b_6/f complex has almost completely disappeared. Similarly, the 53 kDa band in the cytochrome f blot of cross-linked intact b_6/f complex also cannot be detected. These observations thus support our conclusion above that these cross-link products involve the Rieske Fe-S protein. A faint band at about 48 kDa can be observed in both cytochrome f and subunit IV immuno-blots. This might represent a cytochrome f/subunit IV cross-link product. However, the low quantity of this band makes this assignment tentative at present. It is also evident from the cytochrome f immuno-blot that the formation of the cytochrome f dimer is inhibited in $(b_6/f)_{RD}$ complex. This might have been caused by the dissociation of the dimer structure during the detergent treatments on the hydroxyapatite column used to separate the Rieske Fe-S protein. The 36 kDa form of modified cytochrome f is still observed, however.

Other effects of glutaraldehyde on the intact b_6/f complex

The effects of glutaraldehyde treatment on the lower molecular mass polypeptides (10 kDa or less) observed in the intact b_6/f complex is also examined. The $(b_6/f)_{RD}$ complex apparently lacks these peptides (unpublished observations). It thus appears that the samll polypeptides might have been dissociated from the intact complex along with the Rieske Fe-S protein. Fig. 6 shows the expanded desitometric traces of the low-molecular-weight region from the gel lanes used in Fig. 1. Polypeptides of 13 and 9.5 kDa are observed consistently with various preparations of active complex with the 9.5 kDa band more intensely stained than the 13 kDa band. Of these, the 9.5 kDa peptide is extremely sensitive to the cross-linker glutaraldehyde. It practically all disappeared at 0.02% glutaraldehyde. The 13 kDa polypeptide, on the other hand, is resistant to glutaraldehyde modification.

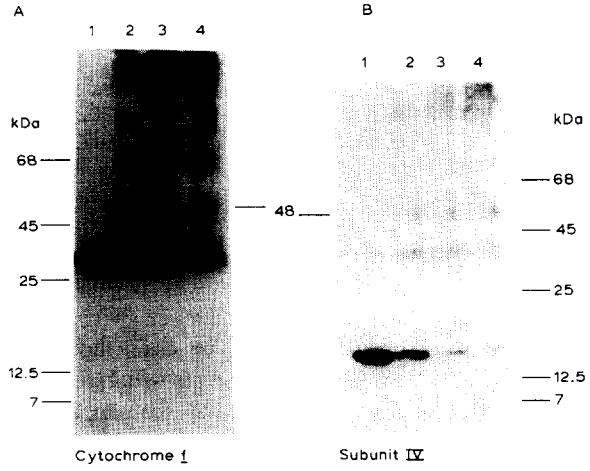


Fig. 5. Immuno-blot analysis of glutaraldehyde-treated Rieske protein-depleted b_6/f complex. Panel (A), spinach cytochrome f antisera; panel (B), maize subunit IV antisera. Other conditions as in Fig. 4.

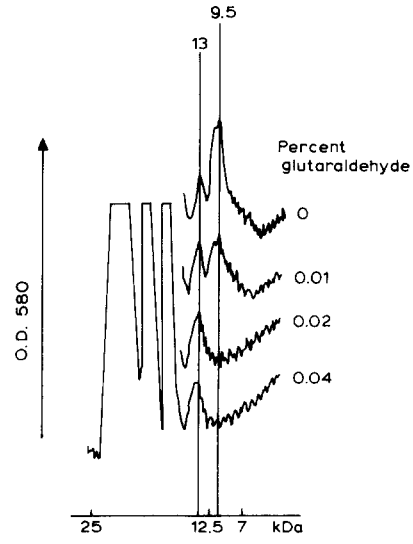


Fig. 6. Effects of glutaraldehyde on the low-molecular-weight peptides of the cytochrome complex. The expanded densitometric scans of gel lanes used for the quantitation of the data for Fig. 1 are shown.

Discussion

Nearest-neighbor relationship of the b_6/f complex

Under the condition of low concentration of reactants and relatively short incubation time, we should be able to minimize random cross-linking due to collision of cytochrome complexes either imbedded in the same detergent micelle or from separate micelles. In the case of the isolated b_6/f complex, the absence of any oligomers from the Rieske Fe-S protein or subunit IV is consistent with this suggestion, since these peptides are relatively exposed as shown by proteolytic digestion studies [5] and the subunit sequence analysis has shown it to be most likely an intrinsic membrane protein also [12]. High frequency of random collision of the complexes should have resulted in oligomers of these polypeptides.

Our observations that the Rieske Fe-S protein is readily cross-linked to subunit IV, while cytochrome b_6 is extremely resistant to cross-linking under the same conditions are in contradiction to the data published by Hauska, G. et al. [5], using a different set of modification conditions. They found that at 0.7% glutaraldehyde, the only subunit significantly modified is cytochrome b_6. It is, however, unclear whether the Tris-succinate buffer present in the cytochrome complex is dialyzed away or not and whether the treatment with sodium borohydride might cleave any bonds formed by the aldehyde functions with the different protein side chains. Since the reaction mechanism of glutaraldehyde is still not well understood [13], it is certainly a possibility that should be considered. In any case, we observed that inclusion of 50 mM Tris-HCl can almost completely inhibit the action of the cross-linker (data not shown). Thus, we add 50 mM Tris-HCl to our samples at the end of the incubation with glutaraldehyde in order to quench unreacted cross-linkers. We then directly solubilize the mixtures for SDS-polyacrylamide gel electrophoresis analysis without further manipulation which eliminates the possible disappearance of some components due to their dissociation by the glutaraldehyde treatment. However, the possibility that the Rieske/subunit IV cross-link product is formed between two b_6/f complexes cannot be ruled out. In view of the observation of cytochrome *f* dimers, this is a possibility that will be examined in the future. Our observation that cytochrome b_6 is most resistant to modification is consistent with it being resistant to proteolytic digest and its hydrophobic nature [5,12]. The observation of a cytochrome *f* dimer is consistent with the conclusion from electron microscopic studies [14], but is in apparent disagreement with gel filtration study with the isolated enzyme complex in 0.2% Triton X-100 which reported a monomeric structure [2]. At present, the apparent discrepancy is unresolved. However, based on the fact that cross-linked cytochrome *f* dimers are formed relatively slowly, it might be suggested that the contact region between the neighboring cytochrome *f*'s is limited in area. The formation of a Rieske/cytochrome *f* product is consistent with them being closely linked in terms of electron-transfer reactions [1]. The fact that in the absence of the Rieske Fe-S protein, cytochrome *f*/subunit IV cross-link products are detected suggests the Rieske Fe-S protein might be situated in a cleft between cytochrome *f* and subunit IV. The observation that the 9.5 kDa peptide is readily cross-linked by glutaraldehyde suggests that either it is a subunit of the b_6/f complex or it exists as oligomers in the b_6/f complex preparation. Identification of the cross-link product from this polypeptide in the future will certainly help in settling this question.

Structure-function relationships among subunits of the b_6/f complex

Our present study bears on the possible function(s) of subunit IV. The co-purification of this subunit has suggested that this polypeptide is a component of the b_6/f complex. However, since no function can yet be assigned to it, the possibility that it is a contaminant rather than a functional component cannot yet be ruled out. Our present observation that it forms a one-to-one cross-link product with the Rieske Fe-S protein is evidence that it is indeed a component of the b_6/f complex. From sequence comparison and hydropathy analysis, Widger, W.R. et al. [6] suggested that subunit IV might have a similar function as that of the COOH terminal of mitochondrial cytochrome *b*. This proposal will predict that the COOH-terminal portion of cytochrome *b* will be cross-linked to the Rieske Fe-S protein in mitochondrial Complex III, in view of the present

work. No such products were reported by Smith and Capaldi [15] in their studies using dithio-containing imidates. In a later study [16], Smith et al. then reported possible cross-linking between the Rieske Fe-S protein and the cytochrome *b* apoprotein, using other reagents that allowed better separation of cross-linked products. In this study, however, it is not certain whether cytochrome c_1 might be the peptide cross-linked to the Rieske Fe-S protein in addition to or instead of cytochrome *b*. Thus, future work with Complex III is needed to confirm and extend the data of Smith et al. and test our prediction above.

Finally, the presence of mitochondrial Complex III in a dimeric form has been concluded from electron-microscopy studies of membrane crystals [17]. Our observation of cytochrome *f* dimers indicates that the b_6/f complex might also exist in a dimeric form, as concluded by Mörschel and Staehelin [14] from freeze-fracture studies of reconstituted b_6/f complex. At present, the functional significance of the dimeric structure of the quinol-cytochrome *c*-type oxidoreductases is unknown. A functional model has been put forth for mitochondrial Complex III [18] in the dimeric form, while none has yet been proposed for the b_6/f complex of chloroplasts.

Acknowledgments

The support and encouragement from Dr. Malkin is gratefully acknowledged. The gift of antibodies from Amy Klecan and Alic Barkan is also appreciated. This work was supported in part by the National Institutes of Health through a grant to Professor Richard Malkin.

References

1 Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133
2 Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599
3 Hurt, E. and Hauska, G. (1982) J. Bioenerg. Biomembranes 14, 405–424
4 Hurt, E., Hauska, G. and Malkin, R. (1981) FEBS Lett. 134, 1–5
5 Hauska, G., Hurt, E., Gabellini, N., Davenport, J.W. and Lockau, W. (1984) in Advances in Photosysnthesis Resarch (Sybesma, C., ed.), Vol. III, pp. 243–249, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
6 Widger, W.R., Cramer, W.A., Herrmann, R. and Trebst, A. (1984) Proc. Natl. Acad. Sci. USA 81, 674–678
7 Capaldi, R.A. (1982) Biochim. Biophys. Acta 694, 291–306
8 Cox, C.F. and Miller, K.R. (1983) Photosynthetica 17, 422–425
9 Lam, E. and Malkin, R. (1982) FEBS Lett. 141, 98–101
10 Chua, N.-H. (1980) Methods Enzymol. 69, 434–446
11 Lam, E., Ortiz, W., Mayfield, S. and Malkin, R. (1984) Plant Physiol. 74, 650–655
12 Heinemeyer, W., Alt, J. and Herrmann, R. (1984) Current Genetics 8, 543–549
13 Papageorgiou, G.C. (1980 Methods Enzymol. 69, 613–625
14 Mörschel, E. and Staehelin, L.A. (1983) J. Cell Biol. 97, 301
15 Smith, R.J. and Capaldi, R.A. (1977) Biochemistry 16, 2629–2633
16 Smith, R.J., Capaldi, R.A., Muchmore, D. and Dahlquist, F. (1978) Biochemistry 18, 3719–3723
17 Leonard, K., Wingfield, P., Arad, T. and Weiss, H. (1981) J. Mol. Biol. 149, 259–274
18 De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 41–53