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Topological analysis of components of the cytochrome b_6f complex by chemical crosslinking

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The cytochrome b_6 *f*-complex from spinach thylakoids has been chemically crosslinked with DSP (3,3′-dithiobis(succinimidylpropionate)), DTBP (dimethyl-3,3′-dithiobis(propionimidate)) and SADP (*N*-succinimidyl[(4-azidophenyl)dithio]propionate). By two-dimensional diagonal SDS-polyacrylamide gel-electrophoresis the following crosslinks were identified: cytochrome *f*-subunit IV, cytochrome *b*-subunit IV and cytochrome *f*-cytochrome *b*. Diepoxybutane did not yield crosslinks. These results suggest that protein domains carrying amino groups are spaced apart for these membrane proteins in a distance of 10–15 Å.

Introduction

The cytochrome b_6f complex of the chloroplast thylakoid membrane plays a central role in the transfer of electrons between the two photosystems; it consists of five polypeptides [1,2]: cytochrome *f* (33 kDa), a two-heme-containing cytochrome b_6 (23 kDa), the Rieske iron-sulfur protein (20 kDa), the so-called subunit IV (17 kDa) and a recently discovered small polypeptide termed subunit V (4 kDa) [3]. Whereas the cytochromes and the Rieske protein participate in electron transport mediated via their functional groups, the role of subunit IV is less defined; by affinity labelling it has been shown recently that subunit IV binds plastoquinone [4]. The function of subunit V is not known.

The determination of the nucleotide sequences of the genes for the cytochrome b_6f components has permitted the deduction of their primary structures. Hydrophobicity analyses identified potential membrane-spanning domains leading to hypothetical models for the arrangement of the proteins in the thylakoid membrane [5]. Protein chemical studies verifying the topology of the membrane components are, however, scarce. Willey et al. [6] applied partial proteolytic digestion to establish the membrane anchorage of cytochrome *f*. Mansfield and Anderson [7], using limited proteolysis and specific antibodies, proposed a stromal orientation of the C-terminal portion of the Rieske protein carrying the putative iron-sulfur cluster. This model is, however, controversial in view of a close association of the Rieske protein with cytochrome *f* [8] and at variance with the membrane orientation deduced from its amino acid sequence [5]. Plastocyanin has been covalently linked to cytochrome *f* using carbodiimide to characterize acidic residues involved in binding [9]. A crosslinking study with glutaraldehyde establishing nearest-neighbor relationships of the constituent polypeptides has been performed by Lam [10]. Here we report crosslinking experiments using cleavable bifunctional reagents of varying molecular length and specificity.

Materials and Methods

Nonanoyl-*N*-methylglucamide (MEGA-9) was obtained from Oxyl (Bobingen, F.R.G.); SDS, Tris buffer, Triton X-100, chemicals and standard proteins for gel electrophoresis were purchased from Serva (Heidelberg, F.R.G.) or Pharmacia/LKB (Freiburg, F.R.G.). Diepoxybutane was from Sigma (Deisenhofen, F.R.G.). The crosslinkers 3,3′-dithiobis(succinimidylpropionate)

Abbreviations: DSP, dithiobis(succinimidylpropionate); DTBP, dimethyl-3,3′-dithiobis(propionimidate); MEGA-9, nonanoyl-*N*-methylglucamide; NEM, *N*-ethylmaleimide; SADP, *N*-succinimidyl[(4-azidophenyl)dithio]propionate; SDS, sodium dodecylsulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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(DSP), dimethyl-3,3′-dithiobispropionimidate (DTBP) and *N*-succinimidyl[(4-azidophenyl)dithio]-propionate (SADP) were obtained from Pierce (distributed in Germany by Bender & Hobein, Munich) and sodium periodate from Fluka (Neu-Ulm, F.R.G.). All other chemicals were from Merck (Darmstadt, F.R.G.). Fresh spinach was purchased from the local wholesale market.

Preparation of cytochrome b_6f complex

The cytochrome b_6f complex was purified according to the method of Hurt and Hauska [1] and Black et al. [11] with the following modifications [8]: (a) prior to extraction with detergent, thylakoid membranes were washed with 10 mM Tricine-HCl (pH 8.0) containing 3 M NaBr and 0.6 M sucrose only once, (b) thylakoid membranes were solubilized in the *dark* with a solution of 40 mM MEGA-9, 0.4 M ammonium sulfate and 0.5–1% sodium cholate (w/v), then centrifuged at 7000 rpm for 12 h, (c) the crude cytochrome b_6f complex was precipitated at 45–55% ammonium sulfate saturation, (d) after hydroxyapatite chromatography with a potassium phosphate gradient towards 0.2 M the cytochrome b_6f complex was concentrated by precipitation with 30% ammonium sulfate and dialyzed overnight at 4°C against 50 mM triethanolamine-HCl (pH 8.0) containing 10 mM MEGA-9 and 0.5% sodium cholate (w/v) or 0.5% Triton X-100 (v/v) instead. This complex solution was stored frozen at −80°C.

Crosslinking and analysis by polyacrylamide gel electrophoresis

Crosslinking experiments were performed in 10–50 mM triethanolamine-HCl (pH 8.0) containing 0.5% Triton X-100 (v/v) or 10 mM MEGA-9 and 0.5% sodium cholate (w/v) as solubilizing detergents. The crosslinking reagents were dissolved in the reaction buffer immediately before use in the following concentrations: diepoxybutane 4% (v/v), DTBP, SADP and DSP 20 mg/ml (DSP and SADP were dissolved in dimethyl sulfoxide). Crosslinking reactions were started by addition of the bifunctional reagents to the cytochrome b_6f complex solutions at room temperature or 37°C; for the determination of the optimal crosslink conditions reagent concentrations were varied as well as reaction times (5–60 min). In the case of SADP the reaction was started by illumination with an ultraviolet lamp for 2 min. Crosslinking reactions were quenched by the addition of 50 mM trimethylamine-HCl (pH 8.0) or 0.36 M glycine containing 5 mM NEM and further incubation for 30 min. For gel electrophoretical analysis the reaction mixture was diluted with the reaction buffer or dialyzed against 15 mM Tris succinate (pH 6.5) containing 15 mM MEGA-9 and 0.25% sodium cholate (w/v); proteins were stained with silver nitrate. For staining with Coomassie Brilliant Blue higher protein concentrations were required. In these cases samples were concentrated prior to gel electrophoresis to approx. 20 mg/ml protein by placing the diluted solutions in a dialysis bag on top of dry saccharose or Sephadex beads for several hours.

One-dimensional slab gel electrophoresis was performed according to Laemmli [12] in a 15% acrylamide gel in 0.38 M Tris-HCl (pH 8.8) containing 0.1% SDS; for the analysis of conjugates containing disulfide crosslinks, reducing agents like mercaptoethanol were omitted. For two-dimensional analysis a narrow strip containing the crosslinked sample was cut out and equilibrated for 1–4 h with 25–50 mM Tris-HCl (pH 6.8) containing 0.2 M glycine, 0.1–4% SDS (w/v) and 5–10% mercaptoethanol (v/v). For the experiments with diepoxybutane crosslinked proteins separated in the first dimension were soaked for 5 h in triethanolamine-HCl (pH 8.0) containing 0.1% SDS, then 4 h in the same buffer containing 15 mM sodium periodate. The gel strip was polymerized with 0.5% agarose and 10% mercaptoethanol on top of a second slab gel and gel electrophoresis was performed as outlined for the first dimension. Proteins were stained with Coomassie Brilliant Blue and silver nitrate according to Oakley et al. [13]. Cytochromes were detected by heme staining according to Thomas et al. [14].

Results and Discussion

Bifunctional reagents that generate crosslinked protein pairs have been used extensively to elucidate protein topologies in supramolecular complexes in the last decades. The use of cleavable reagents (Fig. 1) permits the reversal of crosslink formation and facilitates the identification of crosslinked complexes. A convenient technique for analysis utilizes two-dimensional diagonal gel electrophoresis of the crosslinked complex with an intermediate cleavage step after the first dimension:

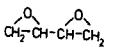
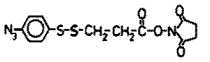
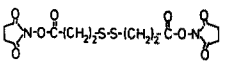
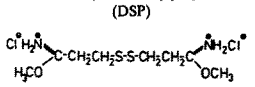
Reagent	Length [Å]	Cleavage
 Diepoxybutane	5	$NaIO_4$
 N-Succinimidyl[(4-azidophenyl)dithio]-propionate (SADP)	8	ß-Me
 3,3'-Dithiobis (succinimidylpropionate) (DSP)	12	ß-Me
 Dimethyl-3,3'-dithiobis(propionimidate)-hydrochloride (DTBP)	15	ß-Me

Fig. 1. Bifunctional cleavable crosslinking reagents. β-Me, β-mercaptoethanol.

binary conjugates of higher molecular weight regain the higher mobility of their constituents in the second dimension yielding protein pairs which migrate off the diagonal.

DSP (3,3′-dithiobis(succinimidylpropionate)) has been applied for the topological analysis of photosynthetic membrane proteins [15–17] leading recently to a specific crosslink of the 33 kDa extrinsic protein with the CP 47 protein in the core of Photosystem II [18]. Application of DSP for crosslinking components of the cytochrome b_6f-complex yields a conjugate at a DSP-concentration of 0.5–7 mM (Fig 2a, lanes 4–7 and 9) with a molecular mass of approx. 40 kDa with a concomitant fading of the constituents with increasing concentrations of the reagent. Staining for heme shows the expected two cytochromes present in the underivatized complex (Fig. 2b) with, in addition, the conjugate observed in the crosslinked sample and cytochrome containing bands in the 54 kDa-range hardly visible in the polypeptide-stained gel strip. Two-dimensional analysis (Fig. 2c). reveals two major crosslinks: a conjugate of subunit IV with cytochrome *b* and another one with cytochrome *f*; the observed molecular masses of 40 kDa and 55 kDa are in good agreement with the expected theoretical values. Thus, crosslinking with DSP yields two defined conjugates.

A cleavable bifunctional reagent with properties similar to DSP is DTBP (dimethyl-3,3′-dithiobis(propionimidate)). Imidoesters have, however, greater amino specificity than the N-hydroxysuccinimide esters. An additional advantage is that the principal product of reaction with primary amines is an amidine that is a stronger base than the original amine; the positive charge of the original amino group is therefore retained. DTBP has also been used for the topology analysis of photosynthetic reaction centers [16]. Application of DTBP for crosslinking membrane proteins of the cytochrome b_6f complex shows, that again a conjugate of molecular mass 40 kDa is formed (Fig. 3a, lanes 2 and 3); upon reduction with 10% mercaptoethanol the crosslink disappears (lane 4). Two-dimensional analysis (Fig. 3b) demonstrates the existence of the same two crosslinks observed with DSP. However, in addition, there is a new crosslink between cytochrome *f* and cytochrome *b* which had not been found in the previous experiments. Since DSP and DTBP have similar chemical and molecular characteristics, it was of interest to test different cleavable crosslinking reagents like the heterobifunctional SADP (*N*-succinimidyl[(4-azidophenyl)dithio]propionate) or a reagent of reduced length like diepoxybutane. SADP has successfully been used for linking the extrinsic 33 kDa protein to core proteins of the photosynthetic center of Photosystem II [19]. Whereas one functional group of this reagent is an amino-specific *N*-hydroxysuccinimide ester, the other is photoactivable yielding an extremely reactive nitrene

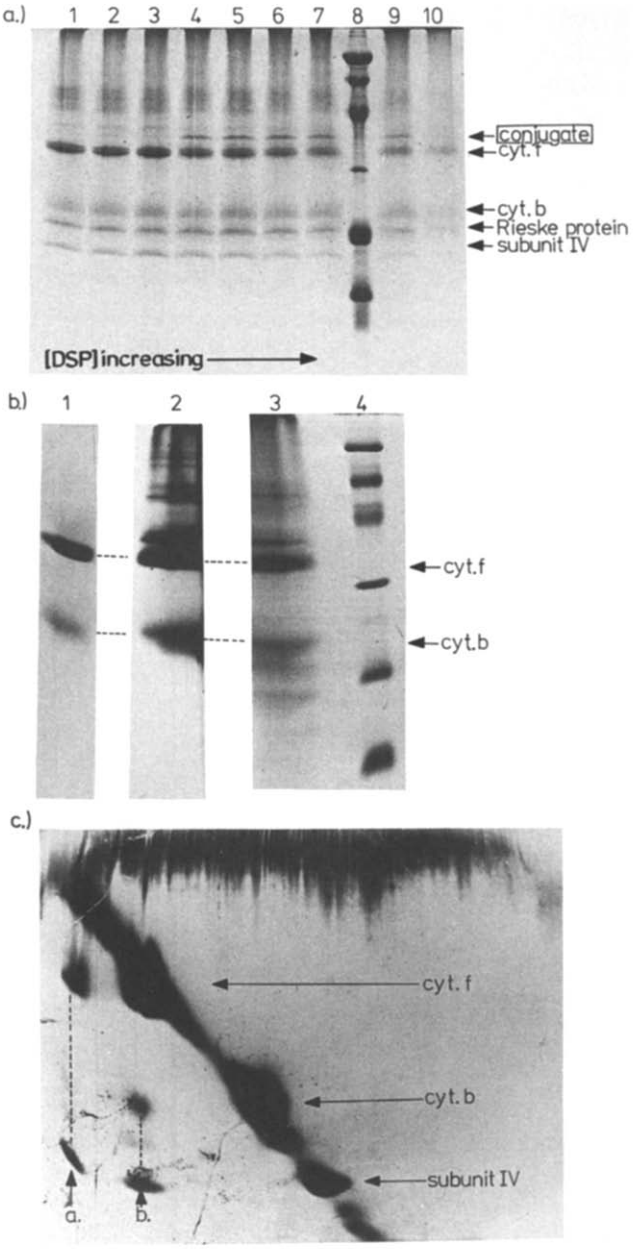


Fig. 2. SDS-polyacrylamide gel electrophoresis of cytochrome b_6f-complex crosslinked with DSP. (a) One-dimensional; lanes 1–7, 9 and 10 with the following DSP-concentrations: 0.05, 0.14, 0.24, 0.48, 1.44, 2.40, 2.70 and 12.0 mM; lane 8: standard marker proteins, Coomassie brilliant blue staining. (b) One-dimensional; lane 1: native cytochrome b_6f complex, heme stained; lanes 2 and 3: cytochrome b_6f complex, crosslinked with 2.4 mM DSP (lane 6 from (a)) and stained for heme (lane 2) and for polypeptides with Coomassie brilliant blue (lane 3); lane 4: standard marker proteins. (c) Two-dimensional diagonal analysis of the crosslinked complex from (a), lane 6; a, cyt *f*-subunit IV crosslink, b, cyt *b*-subunit IV crosslink, silver staining. Reaction conditions: 10 mM triethanolamine-HCl (pH 8.0), 10 mM MEGA-9, 0.5% sodium cholate (w/v), 15 min at 30°C. The reaction was quenched with 0.36 M glycine containing 5 mM NEM.

with broad reaction specificities. Reaction of the cytochrome b_6f-complex with SADP (Fig. 4) leads to a rapid fading of all protein components (lanes 3–6) and

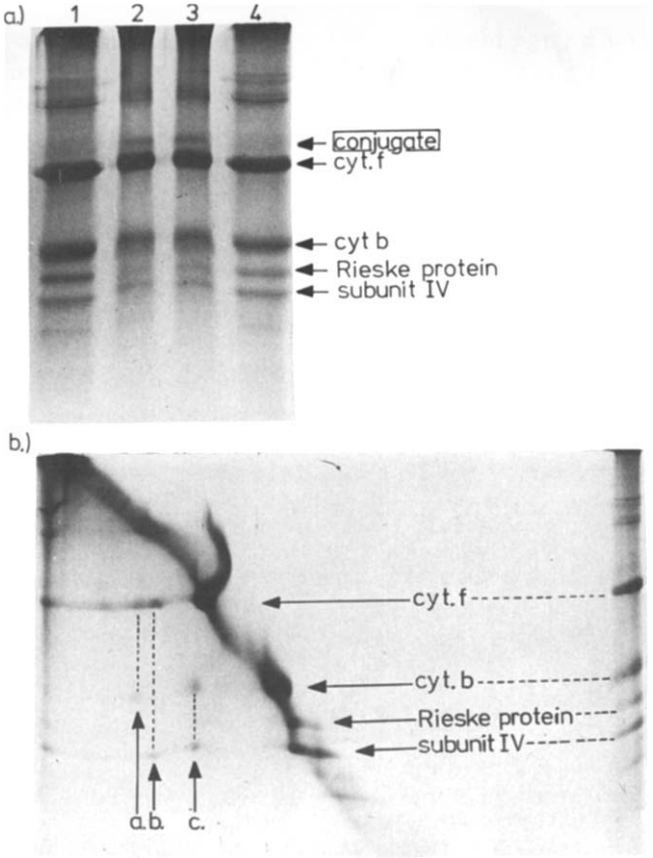


Fig. 3. SDS-polyacrylamide gel electrophoresis of cytochrome b_6f-complex crosslinked with DTBP. (a) One-dimensional; lane 1, underivatized cytochrome b_6f-complex; lanes 2 and 3, crosslinked with DTBP (1 mg/ml); lane 4, crosslinked complex cleaved with 10% mercaptoethanol. (b) Two-dimensional diagonal analysis of the crosslinked complex; a, cyt*f*-cyt*b* crosslink, b, cyt*f*-subunit IV crosslink, c, cyt*b*-subunit IV crosslink. Staining with Coomassie brilliant blue. Reaction conditions: 50 mM triethanolamine-HCl (pH 8.0) 0.5% Triton-X 100, DTBP (1 mg/ml), 30 min at 25°C. The reaction was quenched with 50 mM methylamine-HCl (pH 8.0).

the formation of two higher molecular conjugates. Two-dimensional analysis shows essentially the same crosslinking pattern as observed for DTBP (results not shown); however, in addition, insoluble precipitates are formed and many polymerization products appear obscuring the electrophoretical analysis. Apparently, the high reactivity of the nitrene radical precludes the selective formation of favored binary conjugates.

The short-range reagent diepoxybutane proved valuable for topographical studies for the bacterial ribosome [20–22]. Prolonged treatment of the cytochrome b_6f-complex with 1% diepoxybutane led to a progressive fading of all constituent protein bands (Fig. 4b), the formation of binary or higher conjugates which should yield additional bands was not observed. Higher concentration of diepoxybutane gave the same results. Analysis by two-dimensional diagonal gel electrophoresis showed no proteins located off the diagonal (results not shown). Thus, it is concluded that diepoxybutane produces predominantly monofunctional derivates, and chemical crosslinking apparently does not occur due to the insufficient linker length rather than reactivity, since the reactivity and specificity of the epoxy function is comparable to the activated groups of the other reagents used in this study.

The chemistry of crosslinking suggests the participation of amino groups, e.g. amino terminus or lysine side-chains of the respective membrane proteins. In fact, all components of the cytochrome b_6f complex possess, despite their being embedded in the membrane, polar charged segments which are exposed to the stroma or the thylakoidal lumen. Cytochrome *f* possesses a single hydrophobic membrane-spanning α-helix located near the C-terminus of the polypeptide chain [6] with the more polar domain containing the heme group

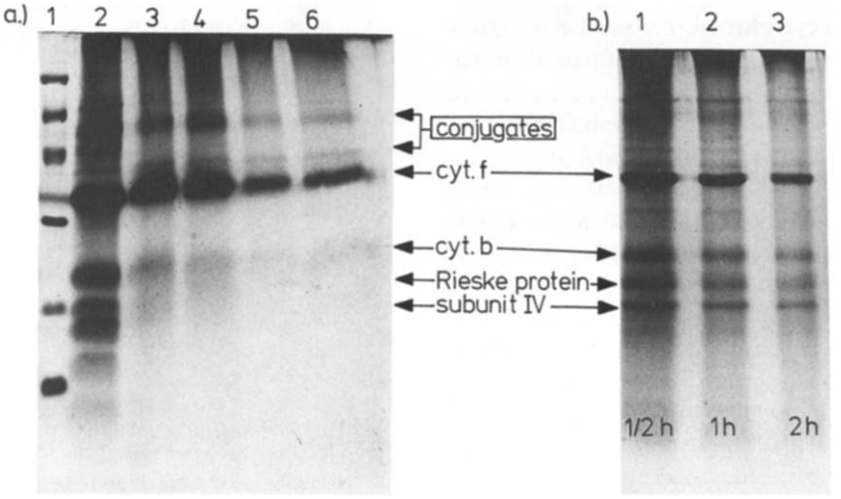


Fig. 4. SDS-polyacrylamide gel electrophoresis of cytochrome b_6f complex reacted with SADP or diepoxybutane. (a) Crosslinking with SADP, lane 1: standard marker proteins, lane 2: underivatized cytochrome b_6f-complex, lanes 3–6: SADP-concentrations 1,2,3 and 4 mg/ml. Reaction conditions: as in Fig. 3, illumination time: 5 min. (b) Time-course of the reaction of cytochrome b_6f complex with diepoxybutane. Reaction conditions: 20 mM triethanolamine-HCl (pH 7.9) 10 mM MEGA-9, 0.5% sodium cholate (w/v), 1% diepoxybutane (v/v), 25°C. The reaction was quenched with methylamine as described in Fig. 3. Staining with Coomassie brilliant blue.

exposed to the thylakoidal lumen. This domain, consisting of about 250 residues, contains 17 lysyl side-chains [23] which could qualify for crosslinking on the lumen side; on the other hand, the presence of four lysine residues on the short stromal extension would likewise permit reactions on this side of the membrane. For subunit IV, models for the arrangement in the membrane with three transmembrane spans have been proposed [24–26] placing the polar C-terminus of the polypeptide in the lumen of the thylakoid membrane; the occurrence of lysyl side-chains on the stromal N-terminal as well as on the lumenal C-terminal segments and in the loop connecting the transmembrane spans offers a variety of crosslinking options with a rather high probability for the N-terminal part carrying a basic cluster of four lysines and one arginine within a stretch of 16 amino acids. Somewhat controversial is the model proposed for the membrane arrangement of cytochrome *b*. Whereas Widger et al. [25] propose five transmembrane α-helical sequences, Willey and Gray [5] opt for four such segments rejecting the membrane span IV postulated by Widger and coworkers: the presence of an aspartate, a glutamate and two or more proline residues makes it according to Willey and Gray very unlikely that this sequence forms a membrane-spanning α-helix. This model places the N- and C-termini both towards the stromal space. This orientation would favor crosslinking with subunit IV on the stromal side since the N-terminal segment contains two and the C-terminal extension one lysine residue. Chloroplast cytochrome *b* and subunit IV show high homology to N- and C-terminal domains of the mitochondrial cytochrome *b*, supporting the theory that their genes arising originally from a common ancestor have been split during evolution [27]. In view of this fact, it is not surprising that a cytochrome *b*-subunit IV crosslink involving possibly the C-terminal part of cytochrome *b* and the N-terminal segment of subunit IV appears to be favored in our studies.

Curiously, the Rieske iron sulfur-protein apparently does not participate in chemical crosslinking, although a strong association with cytochrome *f* has been observed [8]. The reason for this unreactivity is unknown. Possibly, domains qualifying for reaction are either inaccessible or unfavorably oriented.

Using glutaraldehyde, Lam [10] reports crosslinking the Rieske protein with subunit IV and with cytochrome *f* as detected by specific antibodies. These results are not comparable with ours, since glutaraldehyde displays reactivity with several amino acid side chains, including those of lysine, cysteine, histidine and tyrosine. Furthermore, this reagent forms in dilute solution a large number of different polymeric forms precluding a reliable estimation of the distance between cross-linked groups. From the experiments presented in this study we conclude that the three membrane proteins of the cytochrome b_6f-complex – cytochrome *f*, cytochrome *b* and subunit IV – possess in their membrane arrangement amino group carrying domains spaced at a distance of approx. 10–15Å.

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