Neighbouring subunits of CF_0 and between CF_1 and CF_0 of the soluble chloroplast ATP synthase (CF_1–CF_0) as revealed by chemical protein cross-linking

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Neighbouring subunits of CF_0 (I–III) and between CF_0 and CF_1 (α–ε) of the chloroplast ATP synthase have been examined by cross-linking with Cu-phenanthroline (CuP) and bifunctional, cleavable imidoesters. Imidoesters caused cross-links of α-II, β-I, β-II, γ-II, δ-I and ε-III as well as of I_2, I-III, II-III and III_2 of the CF_0 portion. Subunits α-II, β-I, β-II, γ-II and I-I are close enough to form intermolecular cystine bridges upon CuP-catalyzed oxidation of SH groups. The results indicate that: (i) mainly interactions of the CF_1 subunits α, β and γ with the CF_0 polypeptides I and II are required for binding of CF_1 to the thylakoid membrane; (ii) subunit ε interacts directly with CF_0-III; (iii) the CF_0 portion contains a dimer of subunit I; (iv) subunits α and β appear to be structurally non-equivalent within the protein complex.

Chloroplast *ATP synthase* *Cross-linking* *CF_1-CF_0 interaction* *Neighboring subunits*

1. INTRODUCTION

Like its mitochondrial and bacterial counterpart, the chloroplast ATP synthase (CF_1-CF_0) catalyzes the reversible synthesis of ATP, thereby using or forming an electrochemical potential difference of protons across the thylakoid membrane. This enzyme complex consists of two multimeric protein portions, CF_1 and CF_0 [1–3]. CF_1, the portion extrinsic to the membrane, has an M_r of 400000 [4], is like a disk with axes of 8.4 and 16.8 nm [5,6] and contains α_3, β_3, γ, δ and ϵ subunits [7–9]. The 3 catalytically active α/β subunit pairs are arranged in a ring-like structure [10].

Abbreviations: CF_1 and CF_0, catalytic portion and membrane portion of the chloroplast ATP synthase, respectively; CuP, copper phenanthroline; DTBP, dimethyl 3,3′-dithiobispropionimidate; DSP, dithiobis(succinimidyl propionate); 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; MBI, 4-methylmercaptobutyrimidate

Subunits δ and ϵ are not required for binding of CF_1 to the membrane but the latter polypeptide is essential for photophosphorylation and blocks the flow of protons through the membrane portion CF_0 of the ATP synthase [11,12]. The γ subunit undergoes conformational changes during dark-light transitions of the thylakoids [13,14] and seems to coordinate both proton flow through CF_0 [15] and enzyme catalysis of the 3 α/β subunit pairs.

The possible arrangement of subunits and of functionally important sites in CF_1 has been deduced from cross-linking experiments [16] and fluorescence energy transfer measurements [17,18].

In contrast, the function, stoichiometry and organization of subunits belonging to CF_0, the portion of the ATP synthase intrinsic to the membrane, are less well understood despite the fact that CF_0-III represents the dicyclohexylcarbodiimide-binding proteolipid which is indispensable for H^+ translocation through CF_0 [19]. Although the ratio

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of the CF_0 subunits I–III has been determined [7,8], the values obtained were difficult to interpret. To date no information is available on the relationship of CF_0-I and CF_0-II to subunits a and b of the bacterial F_0 portion.

The lack of information about the organization of subunits of the CF_0 portion and the region where CF_1 is bound to CF_0, the latter problem being of extraordinary importance to a better understanding of the link between H^+ translocation and ATP synthesis, has led us to investigate neighbouring subunits of the soluble chloroplast ATP by protein cross-linking.

2. MATERIALS AND METHODS

The method in [1] was used to isolate ATP synthase from broad bean (*Vicia faba*) chloroplast thylakoid membranes. The purified protein preparations containing 1 mg/ml of protein were dialyzed against 50 mM Na phosphate, pH 8.5, 5 mM $MgCl_2$ and 0.1 mM ATP prior to addition of Triton X-100 up to a final concentration of 0.1%. Freshly isolated ATP synthase preparations were used for protein cross-linking at 4°C.

500 μg protein in 0.5 ml of the buffer mentioned were cross-linked with different quantities of CuP, DTBP, DSP and MBI. DTBP and DSP were dissolved at 1 mg in 50 μl dimethyl sulfoxide and defined volumes but less than 30 μl were added to the cross-linking reaction. Cross-linking was allowed to proceed for 8 h and was quenched with 20 μl of 10% ammonium acetate in ethanol. To exclude disulfide exchange after cross-linking, *N*-ethylmaleimide was added to a final concentration of 100 nM.

MBI (1 mg) was dissolved in 50 μl of 50 mM Na phosphate, pH 8.5, and different volumes were added to the protein solution. Cross-linking was allowed to proceed for 2 h before the reaction was quenched with 50 μl of 1 M Tris-glycine, pH 8.0.

For cross-linking with CuP, equal volumes of 136 mM *o*-phenanthroline and 68 mM $CuSO_4$ were mixed just before use and appropriate volumes yielding final concentrations of $\leq$6.8 mM were added to the protein solutions. The reaction was quenched after 2 h by addition of 20 μl of a 10% solution of diethyldithiocarbamate in distilled water. Cross-linked ATP synthases were separated again on sucrose gradients by centrifugation or precipitated immediately with 10 vols ice-cold acetone before sedimentation at 6000 × *g* for 5 min. The samples were dissolved in 50 μl of 50 mM Na borate, pH 8.0, 4% SDS and 10% glycerol and centrifuged at 20000 × *g* for 10 min. The supernatants were separated by 2D-PAGE using a buffer system as in [8]. A 6–18% polyacrylamide slab gel 2 mm wide was used in the first dimension of electrophoresis (13 cm) to separate efficiently subunit aggregates. Equivalent gel strips 4 mm wide were rinsed in 60% ethanol or stained as in [8]. The strips, kept in ethanol until use, were incubated in 50 mM Na borate, pH 8.0, 2% SDS and 5% 2-mercaptoethanol for 30 min and used for the second dimension of electrophoresis. The gel was placed lengthwise across the top of a 9–18% polyacrylamide gel slab and both gels were polymerized together with a boiling solution of 1% agarose in the above buffer. Gel slabs were washed extensively in 60% ethanol before the protein spots were detected by silver staining [20]. A photographic lightening solution was employed to reduce background staining.

3. RESULTS

The chloroplast ATP synthase from *V. faba* thylakoids contains 9 different polypeptides as revealed by SDS-PAGE (fig.1A). The apparent M_r of subunits belonging to the CF_1 and CF_0, α, β, γ, δ, ϵ, I, II_a, II_b and III, were previously determined electrophoretically to be 59000, 55000, 37000, 21000, 16000, 19000, 17000, 16500 and 8000, respectively [2]. The M_r values for ϵ, I and III are thus in accord with those which were calculated from the amino acid sequence of these components [21–23], whereas the true M_r value of the α and β subunits is about 5000 smaller [21]. Remarkably, subunits II_a and II_b are not interconvertible even by reduction of SH groups prior to carboxymethylation. Cross-linking of the soluble ATP synthase by CuP-catalyzed oxidation of intrinsic SH groups and with the bifunctional, cleavable imidoesters DTBP, DSP and MBI has been employed to identify neighbouring subunits between CF_1 and CF_0 and of the CF_0 portion. A low protein concentration but an excess of cross-linkers were used in favour of intramolecular cross-linking. Inter-

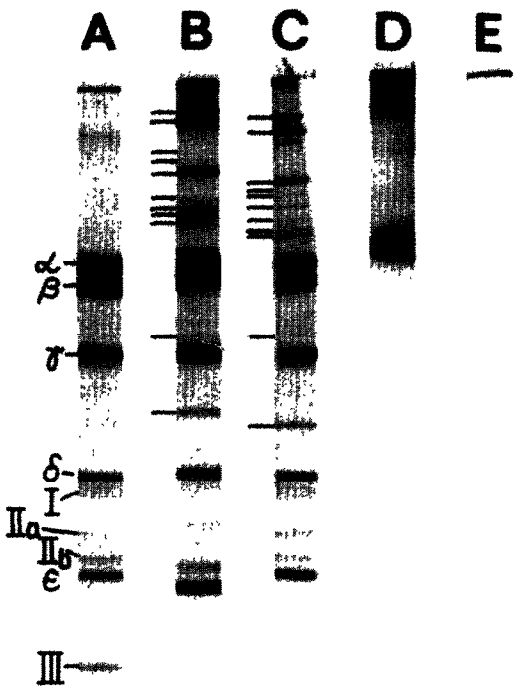


Fig.1. Effect of different cross-linking reagents on the polypeptide pattern of soluble chloroplast ATP synthase. Incubation of the isolated protein complex (A) with 6.8 mM CuP (B), 5 mM DTBP (C), 5 mM DSP (D) and 5 mM MBI (E) and SDS-PAGE using a 6–18% polyacrylamide gel slab were performed as described in section 2. Proteins were stained with Coomassie blue G-250.

molecular cross-linking was negligible because refractionation on sucrose gradients of the cross-linked ATP synthases mostly yielded a single zone of protein as found for untreated material (not shown).

2D-PAGE as described in section 2 was applied to analyze the components of subunit aggregates (fig.2). The control and cross-linked ATP synthases were separated into a diagonal line of subunit spots after the cross-linked polypeptides had been cleaved by 2-mercaptoethanol. Most of the spots beneath the diagonal could be aligned to give the proper pairing and apparent M_r of polypeptide aggregates that were formed by cross-linking. It is worth mentioning that the various subunit aggregates and free subunits of the ATP synthase cross-linked with CuP and DTBP were stained intensely by Coomassie blue fig.1B,C) but only faintly so or not at all for those of the protein complex treated with DSP and MBI (fig.1D,E) when separated on the first dimension gel. It appears, therefore, that dye binding of subunits decreases drastically if DSP and MBI couple to free amino groups.

2D-PAGE of uncross-linked ATP synthase did not resolve subunit aggregates except a small amount of a γ-II dimer (M_r 54000) (not shown). The most unambiguous results of cross-linking were obtained with CuP (fig.2A). Analysis of the off-diagonal spots revealed, in order of increasing M_r, a dimer of subunit I (M_r 38000) and cystine bonds between γ-II_a, γ-II_b (M_r 54000), β-II (M_r 72000), β-I (M_r 74000), α-II (M_r 76000) and β-γ-II_a (M_r 99000) beside complexes containing α, β, γ and ϵ subunits. Dimers of α-ϵ and β-ϵ were also found. The two off-diagonal ϵ spots of M_r 23000 and 25000 are difficult to explain because they do not represent dimers of this subunit.

DTBP has been used as a cross-linking reagent which reacts with primary amino groups and spans 1.2 nm. However, even at high concentrations of 5 mM, cross-linking of ATP synthase subunits with DTBP was not as efficient as with the other imidoesters employed (not shown). Like CuP, DTBP produced dimers of γ-II_a, β-I, β-II_a, β-II_b and α-II but a trimer of β-γ-II was not observed.

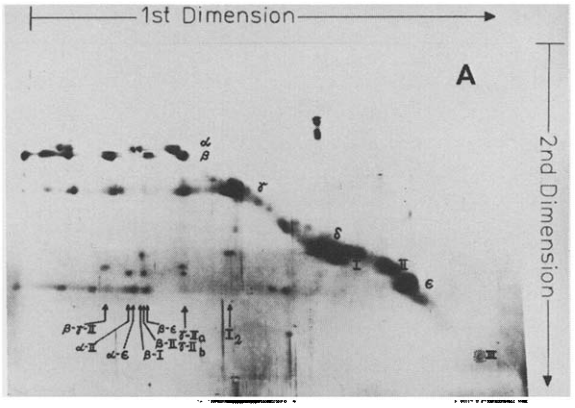
Cross-linking the ATP synthase with DSP, an uncharged imidoester with a span of 1.2 nm that reacts with primary amino groups, caused aggregates containing CF_1 and CF_0 subunits and CF_0 components only (fig.2B). The subunit pairs III_2 (M_r 16000), II-III (M_r 25000), ϵ-III (M_r 24000), δ-I (M_r 40000) and γ-II have been identified with certainty. The proper subunit pairing of cross-links with $M_r > 60000$ was difficult to establish due to overloading of the gel with protein.

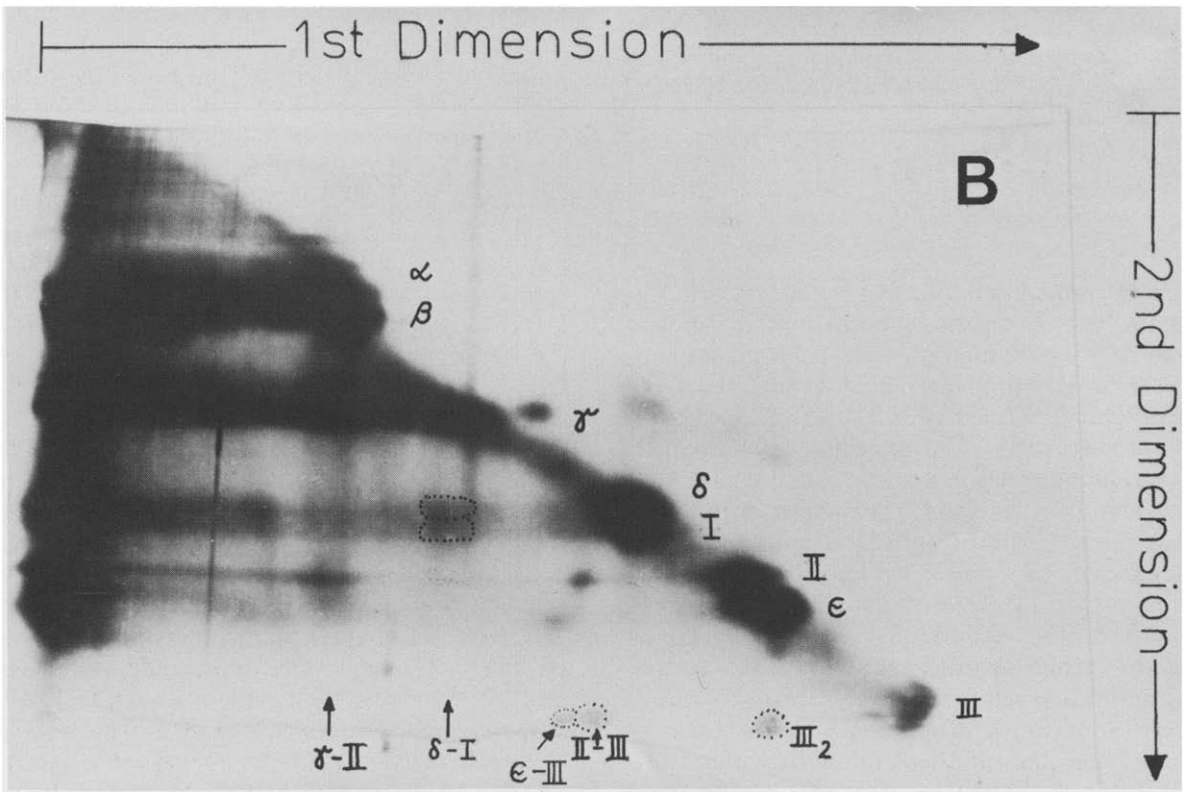
Cross-linking the ATP synthase with MBI produced somewhat different subunit cross-links as seen with DSP (fig.2C). MBI spans 0.8 and 1.6 nm in its reduced and oxidised form, respectively, and reacts with both primary amino and SH groups. 2D-PAGE resolved III_2, I-III (M_r 27000), II-III, ϵ-III, I_2, γ-II_a and γ-II_b dimers as prominent products of MBI cross-linking. As with DSP, the proper pairing of subunits in the aggregates of $M_r > 60000$ was difficult to establish, although the presence of β-ϵ, β-II, β-I dimers is indicated.

4. DISCUSSION

Protein cross-linking has been employed to identify neighbouring subunits of the CF_0 portion and between the catalytic portion CF_1 and CF_0 of the soluble chloroplast ATP synthase. The results indicate that the hydrophilic domains of the CF_0 subunits I and II are close enough with the CF_1 subunits α, β and γ to permit ion-mediated binding interactions. The contact surfaces of the subunit pairs α-II, β-I, β-II and γ-II must possess at least two SH groups, one of each polypeptide not more than 0.6 nm in proximity to allow CuP-mediated cystine formation to occur. In comparison, imidoester cross-linking of the *E. coli* ATP synthase did not indicate neighbour relationships of F_0 polypeptides and the F_1 α and γ subunits and thus it may be concluded from this study [24] that F_1-F_0 binding is solely due to the observed β-F_0-b and β-F_0-a interactions. The reason for the discrepancy between the results in [24] and those here may be either differences in accessibility to cross-linkers of subunits in the soluble and membrane-bound ATP synthases or even more likely species-specific structural differences.

The finding that the chloroplast β subunit which contains only 1 SH group [21] may form cystine links with subunits I, II and ϵ provides the first independent support for different neighbour subunit relationships of the 3 β components within the





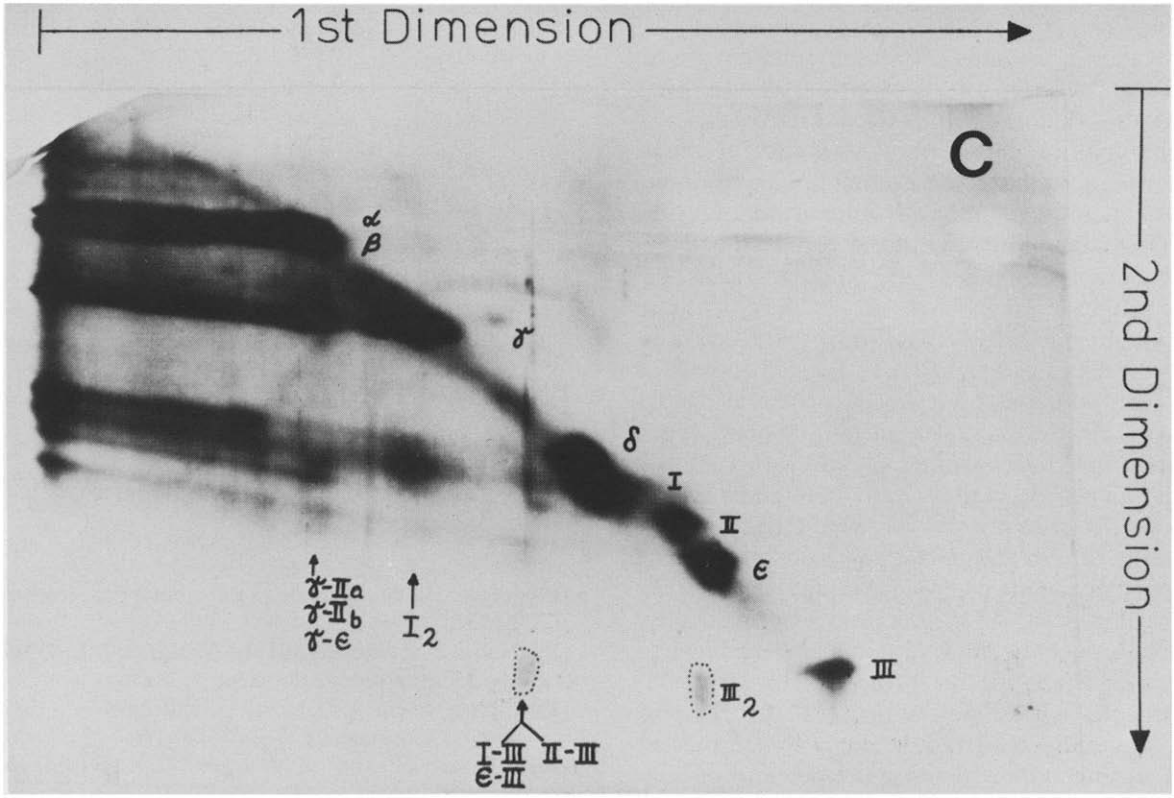


Fig.2. Two-dimensional SDS gel electrophoretic separation of the cross-linked soluble ATP synthase. The enzyme complex treated with CuP (A), DSP (B) and MBI (C) with reagent concentrations as indicated in fig.1 was separated on a 6–18% polyacrylamide gel in the first dimension of electrophoresis. Cross-links were cleaved with 2-mercaptoethanol and the polypeptides separated in the second dimension of electrophoresis using a SDS/5 M urea, 9–18% polyacrylamide gel slab. Proteins were visualized by silver staining. The cross-linked subunits were indicated at the lower end of the gels.

ATP synthase. Furthermore, the 3 α subunits also appear to be structurally nonequivalent because the α subunit contains only 1 SH group [21] but forms cystine links with CF_0-II and ϵ upon CuP-cross-linking. Differences in the organization of α subunits within the CF_1 have also been assumed from fluorescence labeling [18] and proteolysis experiments [25]. In contrast to earlier suggestions [26,27], previous experiments showed the δ and ϵ subunits of CF_1 to be of less importance for binding of CF_1 to the membrane [11,12]. However, subunit ϵ is an absolute requirement for photophosphorylation in that it prevents the leak of protons through the CF_0 [12]. This is in accord with our previous finding for the intact ATP synthase where trypsin digestion of the γ subunit simultaneously led to the disappearance of the ϵ and CF_0-III subunits whilst CF_1 remained bound to CF_0-I and CF_0-II [25]. Subsequently, a correlation between the disappearance of subunits ϵ and III and the complete inhibition of photophosphorylation and concomitant activation of ATPase activity to maximal values has been established [28]. The latter results already indicated the existence of binding interactions among γ, ϵ and CF_0-III subunits. The present cross-linking data support this view and show that at least the ϵ subunit is nearest neighboured to CF_0-III. That a comparable study on the *E. coli* ATP synthase failed to detect a neighbour relationship of the equivalent components remains to be explained. Although binding interactions between α and/or β and F_0-c have been observed by subunit reconstitution of the *E. coli* ATP synthase [29], no

cross-links between these polypeptides could be achieved in a previous [24] and the present study. However, negative results of cross-linking do not prove that such interactions could not exist.

Although the present results are insufficient to construct a model of CF_0, cross-linking with imidoesters and CuP indicates subunit relationships of III_2, II-III and I_2 and thus a compact arrangement of polypeptides of the CF_0 portion as has been proposed for F_0 of the *E. coli* ATP synthase [24,30]. Remarkably, cross-linking with CuP and DSP produced a dimer of CF_0-I which shows for the first time the presence of neighboured SH groups at the contact surface among both subunits. Obviously, since F_0-b also forms a dimer in the *E. coli* ATP synthase [24,30] and CF_0-I is very similar in its primary and predicted secondary structure to F_0-b [22], CF_0-I and F_0-b appear to be functionally equivalent constituents in bacterial and chloroplast ATP synthases. Consequently, although unexpected, CF_0-II might be related to F_0-a of bacteria. If the present results are confirmed, the subunit stoichiometry of the CF_0 portion may be $I_2:II_1:III_6$ (?). However, the present data led the author to revise the previous conclusion that subunits I and II do not belong to the ATP synthase [8].

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