Chemical Cross-linking Studies of Chloroplast Coupling Factor 1*

(Received for publication, June 28, 1976)

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Cross-linking reagents have been used to link covalently adjacent subunits of solubilized spinach chloroplast coupling factor 1, which is a latent ATPase. 1,5-Difluoro-2,4-dinitrobenzene, dimethyl-3,3'-dithiobispropionimidate, and dimethylsuberimidate are able to form bridges of 3 to 11 Å between amino groups, and hydrogen peroxide and the o-phenanthroline-cupric ion complex catalyze the oxidation of intrinsic sulfhydryl groups. The five individual subunit bands (α, β, γ, δ, and ε) and several new aggregate bands can be separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The same four fastest moving aggregate bands, as characterized by their mobilities, migrate more slowly than the heaviest subunit band and appear with all of the cross-linkers employed. The subunit composition of the aggregate bands has been determined through the use of the reversible cross-linkers, dimethyl-dithiobispropionimidate, (o-phenanthroline)Cu(II), and H₂O₂, and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis in which aggregates are separated in the first dimension, the disulfide cross-links are cleaved, and the individual subunits present in the aggregates are separated in the second dimension. The subunits are detected by Coomassie brilliant blue staining and by labeling some of the sulfhydryl groups of the γ and ε subunits with radioactive N-ethylmaleimide. The results obtained indicate that the α and β subunits can cross-link directly with each of the other subunits, that two β subunits are adjacent, and that γ, γ, δ, αδ, and ββ aggregates are present. A minimal subunit stoichiometry consistent with these results is αβγδε. A possible structural model of the coupling factor is derived from the data. Similar, but less extensive, experiments have been carried out with the heat-activated coupling factor (which is an ATPase); no difference in the spatial arrangement of subunits is detected from the two-dimensional gel electrophoresis analysis of the cross-linked aggregates.

The purified coupling factor, CF₁, solubilized from spinach chloroplasts has a molecular weight of 325,000 (1) and contains five subunits referred to as α, β, γ, δ, and ε in order of decreasing molecular weight. Electron microscopy studies have shown solubilized CF₁ to be spherical in shape with a diameter of approximately 90 Å (2). Studies with subunit specific antibodies suggest α and γ subunits are intimately involved in the photophosphorylation activity of CF₁ (3). In an analogous bacterial coupling factor, the δ subunit is required for binding of the enzyme to the membrane (4). As purified, the solubilized CF₁ has latent ATPase activity. The enzyme can be activated by treatment with heat (5), dithiothreitol (6), or trypsin (7). A derivative of CF₁, containing only α and β subunits, has ATPase activity which is insensitive to the ε subunit but which can be abolished by reacting one or two tyrosine groups on the β subunit with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (9). Binding and steady state kinetics experiments (10) have indicated the presence of two tight binding sites on solubilized CF₁ which bind ADP and ATP analogs and a single active site for ATP hydrolysis on the heat-activated enzyme. The tight nucleotide sites appear to act as allosteric conformational switches of the Ca²⁺-ATPase activity. Fluorescence energy transfer measurements have been used to map the distances between specific sites on the α, β, and γ subunits (11-13).

Chemical cross-linking is another approach to studying the organization and interactions of the CF₁ subunits within the intact enzyme. If stable aggregates formed by covalent bonding between adjacent subunits are separated and subsequently identified with respect to their subunit composition, then the subunit composition of the enzyme may be reconstructed from the overlapping parts. The cross-linking of two or more neighboring subunits requires that the necessary functional groups be accessible in proximity and orientation. The length of an artificially introduced bridge determines the distance range...
through which cross-linking can occur. In this investigation the following cross-linking agents of different specificities and distance ranges were employed: F\textsubscript{2}DNB, which is capable of forming a 3 Å bridge between amino groups and tyrosine phenolic groups (14, 15); the bifunctional imidoester, DMS, which can form an 11 Å long bridge between amino groups (16-18); DTP, which is an 11 Å bifunctional imidoester and contains in its carbon chain a disulfide bond that is readily cleaved (17); H\textsubscript{2}O\textsubscript{2}, which was used to facilitate the oxidation of free sulfhydryl groups on the polypeptide chains (18); and CuP, which also cross-links by oxidation of sulfhydryl groups to form a disulfide bond (19, 20). By amino acid analyses (3, 5, 8) the CF\textsubscript{i} subunits have been shown to contain many lysine and tyrosine residues so that there is a high probability of cross-linking among all neighboring subunits with the reagents F\textsubscript{2}DNB, DMS, and DTP. The α, β, γ, and ε subunits have 1.8, 3.1, 5.8, and 1 half-cystine, respectively (3, 8). Disulfide cross-linking facilitated by the reagents CuP and H\textsubscript{2}O\textsubscript{2} is, of course, restricted by the required close proximity of two sulfhydryl groups during the reaction.

The covalently linked CF\textsubscript{i} subunit aggregates have been separated by means of SDS-polyacrylamide gel electrophoresis in two dimensions. Elucidation of aggregate subunit composition was facilitated by the reversible nature of disulfide bonds, those formed between intrinsic sulfhydryls and that contained within the DTP bridge. A minimum subunit stoichiometry of CF\textsubscript{i} and a working model for the structural arrangement of the subunits have been suggested from the results obtained.

**EXPERIMENTAL PROCEDURE**

**Chemicals**—The sources of the chemicals were as follows: F\textsubscript{2}DNB, β-mercaptoethanol, and triethanolamine hydrochloride from Sigma Chemical Co.; DTP from Pierce Chemical Co.; [\textsuperscript{3H}M\textsubscript{al}NE\textsubscript{t}] from New England Nuclear; SDS from Bio-Rad Laboratories; acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine from Eastman Kodak Co.; NCS tissue solubilizer from Amersham/Searle Corp.; and 1,10-phenanthroline from Fisher Scientific Co. The DMS, synthesized from the suberonitrile (16), was a gift from P. M. Searle Corp.; and l,lO-phenanthroline from Fisher Scientific Co. The chemicals were used as supplied except as noted. The N, O, y, and t subunits have 1.8, 3.1, 5.8, and 1 half-cystine, respectively (3, 8). Disulfide cross-linking was facilitated by the reagents CuP and H\textsubscript{2}O\textsubscript{2}, which are restricted by the required close proximity of two sulfhydryl groups during the reaction.

**Preparation of CF\textsubscript{i}**—The CF\textsubscript{i} was prepared by known procedures (21). Fractions with a fluorescence ratio (305:340 nm emission; 280 nm excitation) greater than 1.8 were used in all experiments. The enzyme was stored as an ammonium sulfate precipitate at 4°C. An extinction coefficient of 0.476 ml/mg cm (10) and a molecular weight of 325,000 (1) were used to determine the molar concentration of CF\textsubscript{i}. The heat-activated enzyme (21) was prepared by incubating 3.1 ELM CF\textsubscript{i} with 85% (v/v) in β-mercaptoethanol and approximately 10 μg/ml of CF\textsubscript{i}, pH 8.0, 25°C. In cross-linking experiments, the heat-activated enzyme (21) was dissolved in a small volume of 50 mM sodium phosphate (pH 7.0) and 1 mM EDTA, with 0.1% SDS. Those protein samples without disulfide cross-links (CF\textsubscript{i} reacted with DMS and F\textsubscript{2}DNB and control CF\textsubscript{i}) were treated with 10 μg/ml of CuP, which were stored as an ammonium sulfate precipitate at 4°C in some cases, the enzyme was dialyzed extensively against the cross-linking buffer before reaction rather than passing it through the Sephadex column. Buffer was added to produce a final enzyme concentration of 0.2 to 1.5 μM.

For cross-linking with CuP, the enzyme was in 0.2 M triethanolamine hydrochloride (pH 8.5). DMS was added from a freshly made stock solution to a final concentration of 3.2 mM. After 1 h the reaction was quenched by the addition of ammonium acetate solution to a final concentration of 50 mM.

In the DMS cross-linking reaction, the enzyme was in 0.2 M triethanolamine hydrochloride (pH 8.5). The DMS was added from a freshly made stock solution to a final concentration of 3.7 mM. After an hour, the reaction was quenched by the addition of an equal volume of saturated ammonium sulfate solution which also served to precipitate the enzyme.

For cross-linking with F\textsubscript{2}DNB, the enzyme was in 0.2 M triethanolamine hydrochloride (pH 8.5), 2 mM EDTA. The F\textsubscript{2}DNB was added from a 30 mM stock solution in dimethylsulfoxide to a final concentration of 66 μM. The cross-linking reaction was run for 10 h in the dark to avoid photodecomposition of the F\textsubscript{2}DNB. The reaction was quenched by the addition of an equal volume of saturated ammonium sulfate solution.

For cross-linking by H\textsubscript{2}O\textsubscript{2} oxidation of sulfhydryl groups, the enzyme was in 50 mM triethanolamine hydrochloride (pH 8.0). Aliquots were taken from a stock solution of 13.6 mM o-phenanthroline and 6.8 mM cupric sulfate and diluted to 43 μM CuP in the reaction mixture. After 3 h, the reaction was quenched by the addition of a concentrated EDTA solution to 1 mM.

All cross-linking reactions were carried out at room temperature. After quenching the reaction, the enzyme was either precipitated with saturated ammonium sulfate solution or concentrated to approximately 2 mg/ml (6 μM) with collodion membranes (Schleicher and Schuell Inc.). Protein samples were prepared for polyacrylamide gel electrophoresis immediately after the cross-linking reactions or the enzyme was stored as the ammonium sulfate precipitate at 4°C. In some cases large amounts of the cross-linked protein were prepared for SDS-polyacrylamide gel electrophoresis, aliquots were removed for immediate use, and the remainder was stored at −20°C for subsequent electrophoresis.

The gel band patterns of the stored samples were identical with those run immediately after the cross-linking reaction. When cross-linking involved disulfide formation (H\textsubscript{2}O\textsubscript{2}, DMS, and CuP), the remaining free sulfhydryl groups were alkylated by the addition of a freshly made MalNE\textsubscript{t} solution to 200-fold excess over protein before the enzyme was concentrated.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis at pH 8.3 followed the procedure of Davis (22) except that a stacking gel was not used. The gel was prepared with 6% acrylamide, 0.16% N,N'-methylenebisacrylamide. The gels (8.0 x 0.5 cm) were electrophoresed at pH 8.3 followed the procedure of Davis (22) except that a stacking gel was not used. The gel was prepared with 6% acrylamide, 0.16% N,N'-methylenebisacrylamide. The gels (8.0 x 0.5 cm) were electrophoresed at pH 8.3 followed the procedure of Davis (22) except that a stacking gel was not used. The gel was prepared with 6% acrylamide, 0.16% N,N'-methylenebisacrylamide. The gels (8.0 x 0.5 cm) were electrophoresed at pH 8.3 followed the procedure of Davis (22) except that a stacking gel was not used. The gel was prepared with 6% acrylamide, 0.16% N,N'-methylenebisacrylamide. The gels (8.0 x 0.5 cm) were electrophoresed at pH 8.3 followed the procedure of Davis (22) except that a stacking gel was not used.
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RESULTS

The molecular weights of the individual subunits of CF₁ were determined by the method of Weber and Osborn (24). A plot of the mobility on SDS-system II polyacrylamide gels versus log molecular weight is linear for the standards used: bovine serum albumin, 68,000; catalase, 60,000; ovomucin, 43,000; cytochrome c, 11,700 (23); and aspartate transcarbamylase, 33,000 and 17,000 (25). The positions of the CF₁ subunit mobilities give the molecular weight values: α, 59,000; β, 54,000; γ, 39,000; δ, 20,000; and ε, 13,500. These molecular weights are in good agreement with the values previously reported (3, 26).

The latent CF₁, enzyme was cross-linked using the reagents H₂O₂, CuP, F,DNB, DMS, and DTP as described under "Experimental Procedure." Conditions were used to ensure that only intramolecular cross-linking occurred. The extent of intermolecular cross-linking was determined by polyacrylamide gel electrophoresis of the enzyme with no denaturing agents present before and after the reaction of CF₁ with DTP. The gel of the protein before the addition of cross-linking reagent has one major band and two faint slower moving bands. Their relative amounts as determined by densitometer tracing are in order of decreasing mobility 90.5%, 6.7%, and 2.8%.

The nonlinearity of the absorbance with protein concentration means the percentages of the two impurity bands are upper bounds. If 0.31 μM CF₁ and 3.54 mM DTP are incubated for 30 min, the same three peaks appear with relative amounts, 86.1%, 5.5%, and 8.4%. With 3.1 μM CF₁, 6.5 mM DTP, and a reaction time of 1 h, the peaks have relative amounts, 79.9%, 7.6%, and 12.5%. Subsequent SDS-polyacrylamide gel electrophoresis of the protein cross-linked under the two sets of conditions gives visually identical band patterns. In all cross-linking experiments reported here, the enzyme concentration is between 0.3 and 1.5 μM with a large molar excess of the cross-linking reagent. As indicated by the above results, intermolecular cross-linking is not appreciable under these conditions.

Fig. 1 shows the normal SDS-system I polyacrylamide gel band pattern of CF₁ (Fig. 1a) and the patterns resulting from cross-linking the enzyme with several reagents (Fig. 1b to f). After cross-linking, several new bands representing covalent aggregates of two or more CF₁ subunits are present. As expected from the nature of the cross-links, a greater number and more intense new bands appear on the gels where lysine and/or tyrosine groups have been bridged (Fig. 1d to f). The δ subunit band remains intense when sulfhydryl group oxidants are used as cross-linkers (Fig. 1b and c), whereas the β-band is greatly depleted when lysines are cross-linked. The γ-band is expanded for lysine-linked proteins compared with un-cross-linked and disulfide-linked proteins. In the case of F,DNB (Fig. 1d), two distinct bands are visible at the position of the γ-band and are designated γ₀ and γ₁. Other than in the vicinity of glycerol were added and the slurry stirred to mix and remove air bubbles. The subsequent electrophoresis procedure was the same as for the first dimension gels.

For radioactivity profiles of the gel rods and gel slab strips, the positions of pertinent stained areas were marked with short copper wires. The gels were then frozen with dry ice and cut in 1-mm pieces with a Mickle gel slicer (Brinkmann Instrument Inc.), noting the sections containing wires before removing the markers. The slices were incubated in 1 ml of NCS solubilizer (9:1 NCS:water) in sealed scintillation vials at 50°C overnight. After cooling, 10 ml of scintillation fluid (0.2 g of 2,5-diphenyloxazole and 5.0 g of 1,4-bis[2-(methyl-5-phenyloxazolyl)-benzene in 1 liter of toluene) were added, and the radioactivity was assayed in a Beckman LS-285 liquid scintillation counter.

The following procedure was used for cleaving the disulfide cross-links in the first dimension polyacrylamide gel and carrying out electrophoresis in the second dimension. The cross-linking protein was first run on a 6.0-cm system I polyacrylamide gel rod, then stained and destained as described. The 45% methanol destaining solution caused the dye band to migrate approximately 1 cm. The voltage was turned off between 0.3 and 1.5 FM. The slab gels were removed from the cells by injecting a Mickle gel slicer (Brinkmann Instrument Inc.) noting the sections containing wires before removing the markers. The slices were incubated in 1 ml of NCS solubilizer (9:1 NCS:water) in sealed scintillation vials at 50°C overnight. After cooling, 10 ml of scintillation fluid (0.2 g of 2,5-diphenyloxazole and 5.0 g of 1,4-bis[2-(methyl-5-phenyloxazolyl)-benzene in 1 liter of toluene) were added, and the radioactivity was assayed in a Beckman LS-285 liquid scintillation counter.

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**Fig. 1.** Spectrophotometric traces at 550 nm of the first dimension of Coomassie blue-stained system I SDS-polyacrylamide gels for cross-linking experiments with latent CF1; uncross-linked CF1 (a) and cross-linked with H2O (b), CuP (c), E6DJB (d), DMS (e), and DTP (f). Photographs of representative gels are also shown. Preparations of samples and electrophoresis conditions are described under “Experimental Procedure.” Total protein loaded onto each gel was 50 to 100 µg. The CF1 subunits are designated α, β, γ, δ, and ε. Cross-linked aggregate bands are labeled 1 to 6.

of the γ-band, no new distinct bands migrating faster than α appear with either lysine-linked or disulfide-linked protein. Several new intense bands slower than α are found for all of the cross-linkers and although relative intensities differ, the patterns are similar. In particular, the fastest moving four of these aggregate bands, as identified by their mobilities on SDS system I polyacrylamide gels, appear for all of the cross-linkers. These are designated 1, 2, 3, and 4 in Fig. 1, b to f. In each of the lysine-linked protein gels, two additional major bands appear and are designated 5 and 6 (Fig. 1, d to f). The disulfide-linked protein gels (Fig. 1, b and c) also contain a thin fifth aggregate band with a mobility corresponding to band 5 of the lysine-linked protein gels.

The subunit composition of the aggregate bands was determined by taking advantage of the reversible nature of some of the cross-linking reagents. The aggregates may be separated and identified by means of their mobilities on SDS gels, then cleaving the reagent-oxidized disulfides or those in the DTP bridges breaks each aggregate group into its subunit components which can be separated again on SDS gels. To this end, a modified two-dimensional acrylamide gel electrophoresis technique was utilized (17). As described earlier, the subunits and aggregates in the cross-linked protein are separated on a cylindrical SDS gel rod in the first dimension, the cross-links are cleaved within the gel, and the entire gel is placed lengthwise across the top of an SDS gel slab. During a second electrophoresis, the subunits which were never cross-linked and the aggregates which are not decomposed form a diagonal line of spots. The subunit components cleaved from the aggregates are free to migrate with their normal mobilities vertically downward from their previous position in the aggregate band, thus appearing as off-diagonal spots. Each of these may be identified by comparing its vertical position with the uncross-linked subunits on the diagonal. The two-dimensional technique includes intrinsic standards: each uncross-linked subunit on the diagonal marks the edge of the horizontal strip in which all of the cleaved subunits of that type should be located. Presence of a subunit spot under an aggregate indicates that it was a component in that aggregate. Small impurity bands in the first dimension cannot be confused with...
aggregate bands as they are not cleaved into CF\(_{1}\) subunits. As previously noted (17), if the polyacrylamide slabs have smaller pores than the first dimension rods, then the second dimension spots are more compact. In this work, use of system II polyacrylamide slabs, which contain twice the relative concentration of bisacrylamide in the first dimension system I gels, best achieved this end although the lower molecular weight subunits still smeared slightly horizontally. The change in gel concentration causes the diagonal in the second dimension to be slightly skewed but no ambiguity results.

The possibility exists that one of the smaller subunits may cleave out of an aggregate band and yet not be visualized after a second dimension electrophoresis because it has become too diffuse to stain distinctly with Coomassie blue. To improve the sensitivity of detection, radioactively labeled protein was employed. The sulfhydryl reagent \([\text{H}]\text{MalNET}\) was used to label the \(\alpha, \beta, \gamma,\) and \(\epsilon\) subunits of the intact enzyme. For the conditions given under “Experimental Procedure,” the total incorporation is 0.82 mol of \([\text{H}]\text{MalNET}\)/mol of CF\(_{1}\), with the following distribution among subunits: \(\alpha \beta\), 20%; \(\gamma\), 24%; \(\delta\), <1%; and \(\epsilon\), 56%. When the \([\text{H}]\text{MalNET}\)-modified enzyme is reacted with the cross-linking reagents DTP, F,DNB, and CuP, the first dimension SDS gels have the same band patterns as the unmodified enzyme. Radioactivity profiles of the three cross-linked protein gels show peaks corresponding to some of the subunit and aggregate bands, but there are no counts in the \(\delta\)-band region or where no stained bands had been previously observed. Somewhat less aggregated material is found with CuP as a cross-linker, which is as expected since the \([\text{H}]\text{MalNET}\)–alkylated sulfhydryls may not undergo catalyzed oxidation to form disulfide cross-links.

In Fig. 2 are examples of second dimension polyacrylamide gel slabs of the latent enzyme cross-linked with CuP (Fig. 2a) and DTP (Fig. 2b) and subsequently cleaved. A summary of these and a number of similar gels from different experiments in which the enzyme was cross-linked with DTP, CuP, and \(\text{H}_2\text{O}_2\) is shown by the schematic composite diagram in Fig. 3. It was found that the spot patterns in CuP and in \(\text{H}_2\text{O}_2\) slabs are identical. In the summary no attempt has been made to indicate the relative intensities of the spots, but all of the spots seen consistently on the gels are shown. As indicated, not all of these spots were seen on all of the gels for all samples. Differences between cross-linking the enzyme with the bifunctional imidoester DTP or with CuP or \(\text{H}_2\text{O}_2\)-catalyzed oxidation of intrinsic sulfhydryl groups evident in the first dimension gels (Fig. 1) are reflected further in the second dimension slabs. The \(\delta\) subunit has no sulfhydryl groups and consequently

![Fig. 2. Photographs of the two-dimensional SDS-polyacrylamide gels of latent CF\(_{1}\); cross-linked with CuP (a) and DTP (b). Approximately 100 to 200 \(\mu\)g of protein were run in a gel rod in the first dimension as in Fig. 1. The cross-links were cleaved within that gel which was then laid on top of a gel slab and electrophoresis was carried out in the second dimension. Detailed procedures are given under “Experimental Procedure.” The first dimension migration was left to right; the second dimension migration was top to bottom. Vertical positions of the cleaved subunits in the slabs are indicated. The first dimension gels have been removed.](http://www.jbc.org/content/262/49/9657/F2.large.jpg)

![Fig. 3. Schematic composite diagram of results from the two-dimensional SDS-polyacrylamide gels of cross-linked latent CF\(_{1}\). These conclusions were derived from six different slabs with both DTP and CuP as cross-linkers and from two with \(\text{H}_2\text{O}_2\) as cross-linker. The positions of subunits and uncleaved aggregates are designated on the axes. Subunits cleaving out of DTP cross-linked aggregates are indicated by \(\circ\) and those that cleave out of aggregates formed in the presence of CuP or \(\text{H}_2\text{O}_2\) are indicated by \(\bullet\). \(\circ\) indicates the spot is seen with all cross-linkers. Relative intensities of spots are not shown here but are seen in Fig. 2 and described in the text. A first dimension gel rod of CF\(_{1}\), cross-linked with DTP is shown at the top of the second dimension slab. Aggregate \(\delta\) is not observed in the first dimension gels of the enzyme cross-linked with CuP or \(\text{H}_2\text{O}_2\). Migration in the first dimension gels was left to right and in the second dimension gels, top to bottom.](http://www.jbc.org/content/262/49/9657/F3.large.jpg)
no off-diagonal 6 is found in slabs of CuP and H₂O₂ cross-linked enzyme, whereas 6 spots clearly appear below certain aggregates in the DTP slabs. Compensation was made for the generally less intense aggregate bands in the CuP and H₂O₂ first dimension gel rods by using overloaded gels for the second dimension.

In none of the slabs are off-diagonal spots seen below the diagonal spots of 6, 6, 7, γ, or α. With all three types of slabs, spots corresponding to γ and ε appear below the diagonal β spot (which is usually smeared with the α spot on the diagonal). Under aggregate 1 in slabs of DTP cross-linked enzyme α and β spots appear, with α the more intense cleavage product, and a distinct 6 spot is also observed. With H₂O₂ or CuP as the cross-linker, α and β spots appear equally intense, and faint γ and ε spots are also visible. Although aggregate bands 2, 3, and 4 are fairly close in the first dimension, their second dimension cleavage products are resolved well enough in some slabs for the spots to be separate. Under aggregate 2, α, β, and 6 appear with DTP, CuP, and H₂O₂ as cross-linkers. Under aggregate 3, α, β, and 6 appear in all slabs, but whereas α and β are equally intense with DTP cross-linked enzyme, β is significantly darker with CuP or H₂O₂ as cross-linkers. The 6 spot under aggregate 3 is usually of about the same intensity as that under aggregate 2 for all slabs. A 6 spot consistently appears under aggregate 3 in the DTP slabs. Under aggregate 4, for all types of slabs equally intense α and β spots appear, but there is very little, if any, stain in the 6 region. A distinct 6 spot is observed with the DTP cross-linked protein under this aggregate. Equally dark α and β spots also appear under aggregates 5 and 6 in DTP slabs, and the 6 and 6 regions are lightly stained although usually there are no distinct spots. Under aggregate 5 in the CuP and H₂O₂ slabs is a distinct α spot. A much smaller amount of stain is observed in the 6 and 6 regions. No ε is stained dark enough to observe under any of the aggregates migrating slower than 1 cm in any of the slabs.

With DTP cross-linked, ³H]-MalNEt-labeled enzyme, strips approximately 1 cm wide were cut out of a slab at the 6 and ε levels; similarly, a strip at the 6 level was cut out of a slab with CuP as the cross-linking reagent. These strips were sequentially sliced into 1-mm wide pieces and counted for the ³H-label as described under “Experimental Procedure.” The radioactivity profiles are shown in Fig. 4 and correlate well with the results obtained from the Coomassie blue-stained slabs. In both slabs the number of counts indicates that the 6 and ε subunits are intense cleavage products from an aggregate that migrates with β in the first dimension. With DTP as a cross-linker, the 6 strip (Fig. 4a) has a peak in radioactivity under aggregates 2 and 3, with smaller amounts of labeled 6 present under aggregate 4. In the ε strip (Fig. 4b) large radioactivity peaks are found under aggregates 1 and 4 as well as under β. A significant number of counts are also observed under aggregate 3 but few under aggregate 2. The label indicates the presence of 6 and ε under DTP aggregates 5 and 6. With the CuP cross-linked enzyme (Fig. 4c), the 6 radioactivity peaks under aggregates 2 and 3, with fewer counts under aggregates 1, 4, and 5. With neither of the cross-linking agents do the radioactivity profiles indicate the presence of 6 or ε in regions where no band was previously detected in the first dimension. In particular, no ε aggregate appears to migrate faster than β.

To further check some of the slab results, a variation of the two-dimensional technique was employed (27). In this method a well separated aggregate band in a stained first dimension system I gel rod is excised. The slice then is soaked in the disulfide-cleaving solution and placed on a fresh system II gel rod. In a second electrophoresis some uncleaved aggregate reappears as well as subunit cleavage products which migrate with their normal mobilities and may be identified by comparison with a duplicate system II gel rod on which the uncross-linked protein has been run. This technique was used for the ³H]-MalNEt-modified enzyme cross-linked with DTP and CuP. Results from the studies of aggregate 1 are shown in Fig.
5. Aggregate 1 is well separated from the other bands in the first dimension gels. With CuP cross-linked aggregate 1 (Fig. 5a), a second dimension gel rod reveals the uncleaved aggregate material as a single band and faster migrating subunit cleavage product bands, $\alpha$, $\beta$, $\gamma$, and $\epsilon$. The visual observations are confirmed by the radioactivity profile which shows obvious peaks are associated with the weakly stained $\gamma$- and $\epsilon$-bands. When the first dimension DTP cross-linked aggregate 1 band is run on a second dimension rod (Fig. 5b), the uncleaved aggregate material separates into two closely spaced bands. Subunit cleavage products in the second dimension are a fairly intense $\alpha$-band with lighter $\beta$, $\gamma$, $\delta$, and $\epsilon$-bands. The visual observations are supported by the radioactivity profile which has peaks under the aggregate 1 bands, $\alpha$, $\beta$, $\gamma$, and $\epsilon$. Counts above background in the $\delta$ region are not observed as $\delta$ is not labeled by [H]MalNEt.

The second dimension gel rod method was used with some of the other aggregate bands not as well separated as aggregate 1 in the first dimension. Results from these experiments support the conclusions obtained from second dimension gel slabs for all of the other cross-linked aggregate bands tested: CuP cross-linked bands $\beta$ and aggregate 3, and DTP cross-linked bands $\beta$ and aggregates 3 and 4. As previously found in the gel slabs, the second dimension rods show $\alpha$ and $\beta$ are equally intense cleavage products from aggregate 3 with DTP as a cross-linking agent, while $\beta$ is a significantly more intense component with CuP. From DTP cross-linked aggregate 4, $\alpha$, $\beta$, and $\delta$ cleave out in the second dimension tubes and a light $\gamma$-band also appears, whereas no distinct $\gamma$ spot was previously visualized in the corresponding region in the slabs. Thus, these results suggest a small amount of $\gamma$ is also present in aggregate

4; however, in this case the possibility of contamination from the first dimension aggregate 3 band cannot be eliminated.

As noted above, in DMS and DTP cross-linked protein first dimension gels, the $\gamma$-band is quite broad compared to CuP and H$_2$O$_2$ cross-linked and uncross-linked protein gels, and the F$_2$DNB cross-linked protein gels have two distinct bands at the $\gamma$ position (Fig. 1). Although the DTP second dimension slabs had shown no $\delta$ or $\epsilon$ off-diagonal spots in this area detectable either by Coomassie blue stain or by radioactivity labeling, to further check these results the first dimension $\gamma$-band region was investigated more closely. In one experiment the whole broad $\gamma$-band was cut out of a first dimension gel of DTP cross-linked protein and treated as described above in the second dimension gel rod method. After the second electrophoresis, the upper part of the band becomes significantly more intense, indicating that cross-links have been cleaved, but no faster migrating bands are observable by means of Coomassie blue stain or in a subsequent radioactivity profile. In an experiment with the F$_2$DNB cross-linked protein, the region of the first dimension gel containing the $\gamma$- and $\gamma$'-bands (Fig. 1d) was sliced sequentially for counting of the [H]MalNEt label. The radioactivity profile shows that both bands contain the label in amounts roughly proportional to stain intensity, indicating that neither consists entirely of $\delta$ subunits.

A summary of the results from all of the two-dimensional electrophoresis experiments with cross-linked latent CF$_1$ is presented in Table I.

Heat-activated CF$_1$ was prepared as described under "Experimental Procedure." After removal of the dithiothreitol by dialysis or Sephadex G-25 gel filtration, the activated enzyme was cross-linked with DTP and CuP under the same concentrations and conditions as were used with the latent enzyme. Specific activities were determined for the uncross-linked activated enzyme before and after passing it through the Sephadex column and found to be comparable. The enzyme after the cross-linking reaction with CuP still had significant activity. After cross linking with DTP the enzyme had lost all activity. The cross-linked samples, as well as uncross-linked heat-activated CF$_1$, were run on first dimension system I

<table>
<thead>
<tr>
<th>First dimension band</th>
<th>DTP</th>
<th>CuP or H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon$</td>
<td>$\epsilon$</td>
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<tr>
<td>$\delta$</td>
<td>$\delta$</td>
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<tr>
<td>$\gamma$</td>
<td>$\gamma$</td>
<td>$\gamma$</td>
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<tr>
<td>$\beta$</td>
<td>$\beta$, $\gamma$, $\epsilon$</td>
<td>$\beta$, $\gamma$, $\epsilon$</td>
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<td>$\alpha$</td>
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<tr>
<td>$\alpha$</td>
<td>$\alpha(H), \alpha(L)$, $\delta$, $\epsilon$</td>
<td>$\alpha(H), \alpha(L)$, $\epsilon$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$\beta$, $\gamma$, $\epsilon$</td>
<td>$\beta$, $\gamma$, $\epsilon$</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$\alpha(H), \alpha(L)$, $\epsilon$</td>
<td>$\alpha(H), \alpha(L)$, $\epsilon$</td>
</tr>
<tr>
<td>$\delta$</td>
<td>$\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$</td>
<td>$\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>$\alpha(H), \beta(L)$, $\gamma(L)$</td>
<td>$\alpha(H), \beta(L)$, $\gamma(L)$</td>
</tr>
</tbody>
</table>

The rows are labeled with respect to the bands which appear in the first dimension gel rods following reaction with the specified cross-linking reagents (see Fig. 1 b, c, and f). A subunit significantly more intense than the others cleaving out of the same aggregate band (allowing for differences in molecular weight) is designated by $H$. A much less intense cleavage product is designated by $L$. The entries are described further in the text.
SDS-polyacrylamide gel rods. Comparison of the Coomassie blue-stained gels with the first dimension gels of their latent enzyme counterparts (Fig. 1) indicates no significant differences in band patterns. No new aggregate bands appear below β and the same characteristic aggregate bands, as indicated by their mobilities, appear above α for both the CuP-oxidized and DTP cross-linked protein. Furthermore, no major changes in the relative intensities of the aggregate and subunit bands are observed. The two-dimensional gel slab electrophoresis technique was carried out on the heat-activated enzyme cross-linked with DTP and CuP. In each case, the resulting aggregate cleavage product off-diagonal spot patterns and intensities are identical with those seen in the similarly cross-linked latent enzyme second dimension slabs (Fig. 2). The [3H]MalNEt CF₁ was heat-activated and found to have activity comparable to the unmodified heat-activated enzyme. This enzyme was cross-linked with CuP and DTP. Radioactivity profiles of the second dimension slabs indicate that the presence of γ and ε in the aggregate bands is no different than that seen previously with the [3H]MalNEt-treated latent enzyme (Fig. 4).

DISCUSSION

The F₂DNB, DMS, and DTP all react with free amino groups and form bridges 9 to 11 Å long. The F₂DNB also reacts with tyrosine phenolic groups. The presence of CuP or H₂O₂ facilitates the formation of disulfide bonds which requires the close proximity of two sulfhydryl groups. That reaction of CF₁, with each of these cross-linking reagents results in essentially the same set of aggregate bands on first dimension SDS gels is good evidence that the true molecular geometry of the enzyme is being observed. The greater amount of cross-linked aggregate material with the lysine cross-linkers relative to the intrinsic sulfhydryl cross-linkers is expected. Special note should be made of the fact that ε subunits with a sulfhydryl alkylated by [3H]MalNEt (28) are still able to form disulfide cross-links in the presence of CuP. This indicates the ε subunit must contain more than the single sulfhydryl residue previously reported (9) or, less likely, that cross-linking occurs through other groups. Since the δ subunit has no cysteine residues, the aggregates found to be present after treatment with CuP or H₂O₂ may be considered a subset of those present after reaction of the enzyme with the lysine linking reagents. Other variations in the relative intensities of the aggregate bands are further manifestations of the differences between the individual cross-linking reagents and the reaction conditions but do not alter the basic conclusions.

The best means of determining the subunit composition of the cross-linked aggregates is with cleavable cross-linkers and the two-dimensional gel electrophoresis technique. Then there is no need to rely on the commonly observed linear relationship between the logarithm of the molecular weight and the mobility of the polypeptide chains in SDS-polyacrylamide gel electrophoresis (16, 23). (This relationship, for example, was invalid in the case of intrachain cross-linked γ subunit.) Also, the uncertainties in the molecular weights of the individual subunits are of no consequence. The mobility of each aggregate band indicates a rough molecular weight range which was used to limit the possible combinations of subunits contained in that band. The set of subunits that were observed to cleave out of the band then was used to reconstruct one or more aggregates and thus determine which of the possibilities are present. The further assumptions are made that subunits appear in the second dimension electrophoresis in amounts representative of their amount in the aggregate and are detectable by the analytical techniques employed. It should be noted that the cleavage of cross-links is not quantitative. In the second dimension gel rod method, care must be taken that a single first dimension band is quantitatively excised.

Fig. 6a shows possible combinations of up to three of the CF₁ subunits in a one-dimensional plot of the logarithm of the molecular weight up to a molecular weight of 138,000. As stressed above, a linear relationship is not necessarily valid with cross-linked polypeptide chains, but such a plot is useful in indicating which aggregates may be grouped together and can be compared with the first dimension SDS gels (Fig. 1) and the results from the two-dimensional technique (Figs. 2 to 5 and Table I).

No cross-linked aggregates migrating faster than β are observed (Figs. 1 to 4). In particular no evidence of ε, δ₁, δ₂, or δ₃ is found. Off-diagonal spots for the γ and ε subunits consistently appear under β in the second dimension slabs with DTP, CuP, and H₂O₂ as cross-linkers, while the δ subunit is not detected in that area. This indicates the presence of γ, but not of δ₁ or δ₂ in an aggregate travelling with the α and β subunits in the first dimension. Data indicating cleavage products of the higher molecular weight aggregate bands 1 to 4 separated in the first dimension (Fig. 1) are shown in Figs. 2 to 5 and are summarized in Table I. A summary of the cross-linked aggregates formed based on the results obtained and the considerations presented above is given in Fig. 6b. The sug-

![Fig. 6](http://www.jbc.org/)
gested list includes all possibilities unless the results clearly indicated that they should be eliminated. For example, $\alpha_2\beta$, and $\beta\gamma$ combinations are included if $\alpha$ and $\beta$ are equally intense cleavage products from a given aggregate band. Any aggregate requiring direct cross-linking of $\delta$, $\delta$, $\delta\gamma$, or $\gamma\delta$ is eliminated because these dimers are not found separately. The $\gamma\delta$ aggregate, but not $\gamma\delta$, is considered a possible component of the aggregate 1 band because while $\gamma$ is a pronounced aggregate easily detectable in the second dimension slabs, no evidence of $\gamma\delta$ is found in aggregate 2. Furthermore, less $\gamma$ is cleaved from aggregate 1 relative to aggregates 2 and 3 (Fig. 4) than might be expected if $\gamma\delta$ were present. Thus, in the second dimension gel rods of DTP cross-linked aggregate 1 (Fig. 5b), the less mobile band of the two containing aggregate material probably consists of $\alpha\delta$ and $\beta\delta$, while the faster band contains $\alpha\delta\gamma$, $\beta\delta\gamma$, and $\gamma\delta\gamma$. The subunits that cleave out of these migrate normally. CuP cross-linked aggregate 1 does not contain $\delta$ so that in the second dimension gel rods (Fig. 5c) only the faster aggregate band containing $\alpha\delta\gamma$, $\beta\delta\gamma$, and $\gamma\delta\gamma$ is present, as well as the cleavage products of these. The greater amount of $\alpha$ cleaving out of DTP aggregate 1 (Figs. 2a and 5b) may indicate that $\delta$ cross-links only to that subunit and not to $\beta$. Selection of the possible subunit combinations in aggregate bands 2, 3, and 4 shown in Fig. 6b comes from direct comparison of Table 1 and Fig. 6a. The molecular weight corresponding to aggregate 4 also allows tetramers such as $\alpha\delta\gamma\delta\delta$, (137,000) which would account for the observation that a small amount of $\gamma$ cleaves out of that aggregate band in the second dimension. A plot of the mobility versus the logarithm of the molecular weight from representative first dimension gels using the conclusions of Fig. 6b yields a slightly concave curve. Similar curvature has been seen previously on the same concentration polyacrylamide gels over the molecular weight range examined here (23).

An increasing number of possible aggregates could be present in the high molecular weight bands designated 5 and 6 in Fig. 1. Thus, elucidation of their subunit composition is of little use in determining the steric arrangement of subunits in the enzyme. However, the general properties of the higher molecular weight aggregate bands are consistent with the interpretations proposed. Association of aggregate 5 with trimers of $\alpha$, $\beta$, and $\gamma$, e.g. $\alpha\beta\gamma$ or $\alpha\gamma\delta$, and aggregate 6 with tetramers of the three major subunits, e.g. $\alpha\beta\gamma\delta$, results in a reasonable extrapolation of the mobility versus logarithm of the molecular weight plot derived from the other aggregate bands. Association of aggregates 5 and 6 with combinations that would come from a higher subunit stoichiometry, e.g. $\alpha\beta\gamma\delta\gamma\delta\delta$, requires a much more extreme (less linear) extrapolation. However, the uncertainty in such an extrapolation makes these considerations suggestive but not conclusive.

Demonstrated existence of certain cross-linked aggregates is a reliable indication of the spatial proximity of subunits within the enzyme molecule; however, as similar studies have expressed (20), caution must be exercised in drawing conclusions from negative cross-linking results. The results obtained here indicate a minimum subunit stoichiometry for CF, of $\alpha\beta\gamma\delta\delta\delta$, which gives a total molecular weight of 319,000. The molecular weight found by equilibrium centrifugation is 325,000 (1). Thus, additional $\delta$ and $\epsilon$ subunits are not unreasonable. Additional $\alpha$, $\beta$, and $\gamma$ subunits are unlikely if the molecular weight values of the individual subunits and of CF, are reliable.

A tentative model of the subunit structure of solubilized CF, constructed from the results obtained is shown in Fig. 7. The large amount of $\beta$ subunit cleaving out of aggregate 3 of the disulfide cross-linked protein (Fig. 2a) suggests that two $\beta$ subunits are close together. In this model, the $\alpha$ and $\beta$ subunits are in positions to cross-link with every type of subunit. Although the $\delta$ subunit readily cross-links through amino groups as exhibited by its near depletion in the first dimension gels (Fig. 1 d to f), it cross-links only to $\alpha$ and $\beta$ subunits.

The above interpretation of the cross-linking results is consistent with results from independent investigations of the individual and interrelated roles of $\alpha$, $\beta$, and $\gamma$ subunits in the whole enzyme. The observed stoichiometry of quercetin, 8-anilino-1-naphthalenesulfonate, and nucleotide binding sites on CF, (10-12) is consistent with an $\alpha\beta\gamma$ structure, and the distance determination between several pairs of specific sites on $\alpha$, $\beta$, and $\gamma$ subunits (11-13) can be visualized in terms of the model presented here. Other studies have shown that the $\beta$ subunits contain the catalytic site (or sites) (9), possibly located between them (11). The $\gamma$ subunit has been postulated to provide a binding site for the $\epsilon$ subunit (9). Investigation of the CF, subunits with antibodies has provided information about the location of the subunits (3). The observation that $\gamma$ precipitates the solubilized enzyme but does not cause agglutination of CF, in chloroplast membranes while anti-$\alpha$ and anti-$\beta$ do agglutinate chloroplasts, together with the implication that the $\delta$ subunit is involved in binding CF, to the membrane leads to the suggestion that the $\gamma$ and $\delta$ subunits are on the same side of the enzyme and masked by the membrane. Both this model and our interpretation, that $\delta$ and $\gamma$ subunits are on opposite sides of the enzyme, rely on negative results and therefore are not conclusive. In terms of our model, the $\delta$ and $\gamma$ subunits could both be close to the membrane, yet not close to each other.

Recent investigations of coupling factors isolated from other sources but with many properties similar to CF, have led to different conclusions concerning subunit structure. A stoichiometry of $\alpha\beta\gamma\delta\delta\delta$ has been proposed for beef heart mitochondrial ATPase on the basis of radioactive labeling of sulfhydryl groups (29). The structure $\alpha\beta\gamma\delta\delta\delta$ was concluded to be present in bacterial coupling factors from $^{14}$C-labeled amino acid incorporation experiments and accompanying cross-linking experiments were interpreted as suggesting that $\alpha$ and $\beta$ subunits were arranged alternatively as a planar hexagon about the central $\gamma$ subunit (30). The structure $(\alpha, \gamma, \epsilon)(\beta, \delta)\delta\delta$ is a tetrameric arrangement was suggested.
for the bacterial coupling factor from analysis of fragments produced by freezing the solubilized enzyme in salt solutions (31). Of course, the subunit stoichiometry and arrangement need not be identical for all coupling factors. They have yet to be established for any coupling factor.

The heat activation of solubilized CF$_1$ ATPase in the absence of detergents has been suggested to involve movement of the $\alpha$ subunit(s), possibly to the membrane binding site, thereby causing removal of the inhibition with simultaneous loss of coupling activity (8). Our results indicate that the arrangement of subunits as revealed by cross-linking experiments is not greatly different in the latent and heat-activated enzyme.

In summary, the use of chemical cross-linking has provided a minimum subunit stoichiometry ($\alpha_2\beta_2\gamma_2\delta$) and a working model (Fig. 7) for CF$_1$, which now can be tested by other experiments.

Acknowledgment—We gratefully acknowledge the technical assistance of Miss Jean Hsu.

REFERENCES

Chemical cross-linking studies of chloroplast coupling factor 1.
B A Baird and G G Hammes


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