# Rotation of Nucleotide Sites Is Not Required for the Enzymatic Activity of Chloroplast Coupling Factor 1†

Karin M. Musier‡ and Gordon G. Hammes*
*Department of Chemistry, Cornell University, Ithaca, New York 14853-1301*
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ABSTRACT: New heterobifunctional photoaffinity cross-linking reagents, 6-maleimido-*N*-(4-benzoylphenyl)hexanamide, 12-maleimido-*N*-(4-benzoylphenyl)dodecanamide, and 12-[$^{14}$C]maleimido-*N*-(4-benzoylphenyl)dodecanamide, were synthesized to investigate the mechanism of ATP hydrolysis by chloroplast coupling factor 1. These reagents react with sulfhydryl groups on the $\gamma$-polypeptide. Subsequent photolysis cross-links the $\gamma$-polypeptide covalently to $\alpha$- and $\beta$-polypeptides. The cross-linkers prevent major movements of the $\gamma$-polypeptide with respect to the $\alpha$- and $\beta$-polypeptides but are sufficiently long to permit some flexibility in the enzyme structure. When ~50% of the $\gamma$-polypeptide was cross-linked to $\alpha$- and $\beta$-polypeptides, a 7% loss in ATPase activity was observed for the longer cross-linker and a 12% loss for the shorter. These results indicate that large movements of $\alpha$- and $\beta$-polypeptides with respect to the $\gamma$-polypeptide are not essential for catalysis. In particular, rotation of the polypeptide chains to create structurally equivalent sites during catalysis is not a required feature of the enzyme mechanism.

Chloroplast coupling factor 1 ($CF_1$)[1] is the extrinsic portion of an integral membrane protein that catalyzes ATP synthesis. Isolated $CF_1$ is a water-soluble, latent ATPase of molecular weight 400000 (Moroney et al., 1983) and is comprised of five polypeptides, $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ in order of decreasing molecular weight. The stoichiometry of the polypeptide chains is probably $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983). Three nucleotide binding sites have been found, and their locations are thought to be primarily on the $\beta$-polypeptide, near the interface of $\alpha$- and $\beta$-polypeptides (Bruist & Hammes, 1981; Kambouris & Hammes, 1985; Admon & Hammes, 1987). Furthermore, the $\alpha$- and $\beta$-polypeptides probably are arranged alternately in a ringlike structure about the three smaller polypeptides (Akey et al., 1983). The overall enzyme structure is asymmetric, and therefore, the nucleotide sites are not structurally equivalent [cf. Snyder and Hammes (1984)].

Structural information about $CF_1$ has been obtained from cross-linking experiments (Baird & Hammes, 1976) and from fluorescence resonance energy transfer studies [cf. Snyder and Hammes (1984, 1985) and Richter et al. (1985)]; however, the molecular mechanism of catalysis and regulation of $CF_1$ is still controversial. Existing kinetic data are consistent with at least three classes of mechanisms (Leckband & Hammes, 1987): (1) two-site positive cooperativity, (2) parallel catalytic pathways (Hammes, 1982), and (3) the binding change (alternating site) mechanism [cf. Boyer and Kohlbrenner (1981)]. The possibility of equivalent catalytic sites, as proposed in the last mechanism, is difficult to reconcile with the asymmetric structure of the enzyme. The suggestion has been made, however, that structural equivalence of the nucleotide binding sites occurs during catalysis through rotation of the $\alpha$–$\beta$-polypeptides about the $\gamma\delta\epsilon$ core (Gresser et al., 1982). This model is tested in this work through the use of new bifunctional photoaffinity cross-linking reagents that prevent polypeptide chain rotation while maintaining enzymatic activity. In the past, the use of bifunctional cross-linking reagents has been an important approach to studying the organization of protein subunits (Wold, 1972; Baird & Hammes, 1976). More recently, photoaffinity cross-linkers have proven to be very useful for the study of biological molecules (Galardy et al., 1974; Bayley & Knowles, 1977; Ji, 1979; Moreland et al., 1982; Tao et al., 1985). The major advantage of photoaffinity probes relative to conventional affinity labels is their ability to remain chemically inert until photolysis and the nonspecificity of their covalent bonding interactions.

In this work, two photoaffinity probes, 6-maleimido-*N*-(4-benzoylphenyl)hexanamide (6-MB) and 12-maleimido-*N*-(4-benzoylphenyl)dodecanamide (12-MB), were synthesized and used in cross-linking studies with the activated $CF_1$. Additionally, [$^{14}$C]12-MB was synthesized to enable accurate quantitation of the extent of cross-linking. The results obtained indicate that significant cross-linking (~50%) of the $\gamma$-polypeptide to $\alpha$- and $\beta$-polypeptides occurs with only small losses in the ATPase activity of $CF_1$ (7–12%).

## Materials and Methods

*Materials.* The sources of the chemicals were as follows: ATP (vanadium free), dithiothreitol, acrylamide, *N,N,N′,N′*-tetramethylethylenediamine, $\alpha,\alpha$-dichloromethyl methyl ether, 4-chloro-1-naphthol, dicyclohexylcarbodiimide, trifluoroacetic acid, high molecular weight standards, and *N*-ethylmaleimide from Sigma Chemical Co.; *N*-[$^3$H]ethylmaleimide from New England Nuclear; [2,3-$^{14}$C]maleic anhydride from Amersham Searle; SDS and *N,N′*-methylenebis(acrylamide) from Bio-Rad Laboratories; 6-aminohexanoic acid, maleic anhydride, 4-aminobenzophenone, *N*-methylmorpholine, *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl) thiolcarbonate, and 12-aminododecanoic acid from Aldrich Chemical Co.; goat anti-rabbit IgG antibody from ICN Immunobiologicals; dimethyl suberimidate, dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride, succinimidyl (4-maleimidomethyl)cyclohexane-1-carboxylate, and succinimidyl

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[1] Abbreviations: $CF_1$, chloroplast coupling factor 1; EDTA, ethylenediaminetetraacetic acid; 6-MB, 6-maleimido-*N*-(4-benzoylphenyl)-hexanamide; 12-MB, 12-maleimido-*N*-(4-benzoylphenyl)dodecanamide; [$^{14}$C]12-MB, 12-[$^{14}$C]maleimido-*N*-(4-benzoylphenyl)dodecanamide; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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(4-maleimidophenyl)butyrate from Pierce Chemical Co.; Rexyn 201 ($OH^-$) ion-exchange resin from Fisher Scientific; and 4-maleimidobenzophenone and fluoresceinmaleimide from Molecular Probes, Inc. Ethyl acetate was dried over 3-Å activated molecular sieves before use. All solvents were analytical grade, and all other chemicals were high-quality commercial grades. Solutions were prepared from deionized water.

Silica gel TLC plates with fluorescent indicator were from Macherey–Nagel. Nitrocellulose sheets (0.45 μm) were from Schleicher & Schuell. Melting points were determined with a Fisher–Johns melting point apparatus and are uncorrected. Proton magnetic resonance spectra were obtained on a Varian XL200 spectrometer. Samples were dissolved in $CDCl_3$ or dimethyl-$d_6$ sulfoxide, and chemical shifts are expressed in ppm relative to tetramethylsilane. Electron-impact (70 eV) mass spectra were obtained with an Associated Electronics Industries MS-902 spectrophotometer. Visible and ultraviolet absorbance measurements were made with a Cary 118C recording spectrophotometer. Polyacrylamide gels were sliced with a Brinkmann Instruments gel slicer, and scintillation counting was performed with a Beckman LS1801 scintillation counter.

*Preparation of 6-MB.* 6-Maleimidohexanoic acid chloride was prepared by first refluxing 6-aminohexanoic acid (5.20 g, 40 mmol) and maleic anhydride (4.00 g, 40 mmol) in xylenes (40 mL) under a Dean–Stark water separator (Young et al., 1982). The vapor temperature reached ca. 138 °C, and the reaction was allowed to reflux for 7–8 h. The reaction was monitored by TLC (product $R_f$ 0.60, methanol–chloroform, 1:8 v/v). The TLC plates were visualized under ultraviolet light and developed with bromocresol green. The reaction mixture was cooled and diluted with an equal volume of chloroform. Solvent was removed to yield a white residue (2 g) which was dissolved by gentle heating in 20 mL of 5% sodium bicarbonate. Following extraction with diethyl ether (3 × 10 mL), the aqueous layer was acidified to pH 2–3 with concentrated HCl. The acid was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were dried over activated molecular sieves and reduced to dryness to yield 6-maleimidohexanoic acid, mp 80–81 °C. Low yields of product (0.9 g) were apparently due to a concurrent polymerization (Coleman et al., 1959): $^1H$ NMR ($CDCl_3$) δ 10.1 (1 H, s, COOH), 7.3 (2 H, s, maleimide CH), 3.48 (2 H, t, $NCH_2$–), 2.3 (2 H, t, $-CH_2CO_2$–), and 1.6 (6 H, m, $-CH_2$–).

6-Maleimidohexanoic acid (100 mg, 0.46 mmol) and α,α-dichloromethyl methyl ether (300 μL, 3.00 mmol) were heated at reflux under nitrogen in anhydrous dichloromethane (2 mL) for 12 h. The solution was cooled and concentrated under a slow stream of nitrogen to give a quantitative yield of crude 6-maleimidohexanoic acid chloride. This material, a yellow oil, was used without further purification: $^1H$ NMR ($CDCl_3$) δ 6.7 (2 H, s, maleimide CH), 3.5 (2 H, t, $NCH_2$–), 2.92 (2 H, t, $-CH_2COCl$), 1.6–1.8 (4 H, m, $-CH_2CH_2CH_2$–), and 1.34 (2 H, m, $-CH_2CH_2CH_2$–).

6-Maleimidohexanoic acid chloride and 4-aminobenzophenone were coupled as follows. To a solution of 6-maleimidohexanoic acid chloride (108 mg, 0.44 mmol) in ethyl acetate (2 mL) under argon was added a solution of 4-aminobenzophenone (95 mg, 0.48 mmol) in ethyl acetate (1 mL) with a dry syringe (Yalpani & Hall, 1981). A white precipitate formed. *N*-Methylmorpholine (34 μL, 0.48 mmol) was added, and the mixture was heated under a gentle reflux with stirring for 4 h. The reaction mixture was cooled, filtered, and concentrated under a slow stream of dry nitrogen. The resulting yellow oil was a mixture of products as determined by TLC (toluene–ethyl acetate, 2:1 v/v; phosphomolybdic acid visualization). The major product ($R_f$ 0.30) was purified by silica gel column chromatography (toluene–ethyl acetate, 4:1 v/v) to yield the desired product, 6-MB, as a white solid (44 mg): mp 136–138 °C; $^1H$ NMR ($CDCl_3$) δ 7.6 (9 H, m, aromatic), 6.68 (2 H, s, maleimide CH), 3.54 (2 H, t, $NCH_2$–), 2.4 (2 H, t, $-CH_2CO$–), and 1.6 (6 H, m, $-(CH_2)_3$–); mass spectrum molecular weight, 390 ($M^+$).

*Preparation of 12-MB.* 12-Maleimidododecanoic acid was prepared by refluxing maleic anhydride (1.00 g, 10 mmol) and 12-aminododecanoic acid (2.15 g, 10 mmol) in xylenes (25 mL) under a Dean–Stark water separator. The vapor temperature reached ca. 146 °C, and the reaction was allowed to reflux 3–4 h. Reaction progress was monitored by TLC (product $R_f$ 0.63, methanol–chloroform, 1:8 v/v). After being cooled, the reaction mixture was diluted with an equal volume of chloroform and concentrated under vacuum. The desired product was isolated by partitioning the resulting orange oil between 5% sodium bicarbonate and diethyl ether. The organic fraction was concentrated to yield 12-maleimidododecanoic acid (200 mg) as a white solid: mp 77–80 °C; $^1H$ NMR ($CDCl_3$) δ 6.7 (2 H, s, maleimide CH), 3.5 (2 H, t, $-NCH_2$–), 2.34 (2 H, t, $-CH_2CO$–), and 1.4 (18 H, m, $-(CH_2)_9$–).

Dry 12-maleimidododecanoic acid (130 mg, 0.44 mmol) and α,α-dichloromethyl methyl ether (300 μL, 3.00 mmol) were heated at reflux under nitrogen in anhydrous dichloromethane (2 mL) for 12 h. The solution was cooled and concentrated under a gentle flow of nitrogen to provide the theoretical yield of the crude acid chloride, which was used as a yellow oil without further purification: $^1H$ NMR ($CDCl_3$) δ 6.7 (2 H, s, maleimide CH), 3.5 (2 H, t, $-NCH_2$–), 2.87 (2 H, t, $CH_2COCl$), and 1.4 (18 H, m, $-(CH_2)_9$–).

The coupling of 12-maleimidododecanoic acid chloride and 4-aminobenzophenone was carried out as described for the preparation of 6-MB with the same TLC system to monitor reaction progress (product $R_f$ 0.58). After 4 h, the reaction mixture was cooled and filtered. Following washes with 5% HCl (3 × 3 mL) and water, the organic layer was dried over magnesium sulfate. Purification was achieved by silica gel column chromatography (toluene–ethyl acetate, 5:1 v/v) to yield the desired product, 12-MB, as a white solid: mp 114–118 °C; $^1H$ NMR ($CDCl_3$) δ 7.8 (9 H, m, aromatic), 6.7 (2 H, s, maleimide CH), 3.51 (2 H, t, $NCH_2$–), 2.4 (2 H, t, $CH_2CO$–), and 1.5 (18 H, m, $-(CH_2)_9$–); mass spectrum molecular weight, 475 ($M^+$).

*Preparation of [$^{14}C$]12-MB.* *N*-(*tert*-Butyloxycarbonyl)-12-aminododecanoic acid was prepared by adding *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl) thiolcarbonate (1.32 g, 5.50 mmol) to a solution of 12-aminododecanoic acid (1.08 g, 5.00 mmol) and triethylamine (1.05 mL, 7.50 mmol) at 70–80 °C in 60 mL of 50% aqueous dioxane (Nagasawa et al., 1973). The mixture was maintained at 70–80 °C with stirring and monitored by TLC (product $R_f$ 0.52, hexanes–ethyl acetate, 1:1 v/v). After 3 h, the reaction mixture was cooled, diluted with 100 mL of water, and acidified to pH 3 with cold 5% HCl. The resulting mixture was extracted with ethyl acetate (3 × 50 mL). The ethyl acetate layers were combined, dried over anhydrous sodium sulfate, and concentrated. The crude product was subjected to silica gel column chromatography (hexanes–ethyl acetate, 4:1 v/v) to obtain pure *N*-(*tert*-butyloxycarbonyl)-12-aminododecanoic acid: $^1H$ NMR ($CDCl_3$) δ 4.5 (1 H, s, NH), 3.1 (2 H, t, $-NCH_2$–), 2.34 (2 H, t, $-CH_2CO$–), 1.6 (2 H, t, $-CH_2$–), 1.4 (9 H, s, $(CH_3)_3C$–), and 1.25 (16 H, s, $-(CH_2)_8$–).

*N*-(*tert*-Butyloxycarbonyl)-12-aminododecanoic acid (0.60 g, 1.98 mmol) was dissolved in ethyl acetate (20 mL). To this solution was added dicyclohexylcarbodiimide (0.49 g, 2.38 mmol) followed by 4-aminobenzophenone (0.39 g, 1.98 mmol). The reaction mixture was heated at reflux for 12 h and monitored by TLC (product $R_f$ 0.47, hexanes–ethyl acetate, 1:1 v/v). After the mixture was cooled, the precipitated dicyclohexylurea was removed by filtration. The solvent was removed and the product purified by silica gel chromatography (hexanes–ethyl acetate, 6:1 to 1:1 v/v). After recrystallization from diethyl ether, pure 12-*N*-(butyloxycarbonyl)-*N*-(4-benzoylphenyl)dodecanamide was obtained: $^1$H NMR ($CDCl_3$) $\delta$ 7.65 (9 H, m, aromatic), 4.5 (1 H, s, NH), 3.1 (2 H, t, $-NCH_2-$), 2.4 (2 H, t, $-CH_2CO-$), 1.5 (18 H, m, $-(CH_2)_9-$), and 1.4 (9 H, s, $(CH_3)_3C-$).

12-*N*-(Butyloxycarbonyl)-*N*-(4-benzoylphenyl)dodecanamide (210 mg, 0.43 mmol) was dissolved in methylene chloride (10 mL). The solution was cooled to 0 °C, and 1 mL of trifluoroacetic acid was added. This mixture was stirred at 0 °C for 40–60 min. After the solvent was removed, the crude amine salt was dissolved in methanol and converted to the free base by passage through an activated Rexyn 201 ($OH^-$) column. Pure 12-amino-*N*-(4-benzoylphenyl)dodecanamide was obtained after recrystallization from hexanes–tetrahydrofuran (1:1): $^1$H NMR (dimethyl-$d_6$ sulfoxide) $\delta$ 7.65 (9 H, m, aromatic), 2.36 (2 H, t, $-CH_2CO-$), and 1.35 (20 H, m, $-(CH_2)_{10}-$).

12-Amino-*N*-(4-benzoylphenyl)dodecanamide (39.4 mg, 100 $\mu$mol) was dissolved in tetrahydrofuran (0.8 mL). [2,3-$^{14}$C]Maleic anhydride (16 Ci/mol; 1.5 mg, 15.6 $\mu$mol) was added at room temperature and stirred for 30 min (Tao et al., 1985). Nonradioactive maleic anhydride (9.8 mg, 100 $\mu$mol) was then added. After 1 h, the solvent was evaporated under argon. Anhydrous sodium acetate (100 mg) and acetic anhydride (1 mL) were added to the crude maleamic acid. The mixture was heated at 100 °C for 1 h. Ice water (2 mL) was then added to the reaction flask, and stirring was continued for 2 h at 0 °C (Mehta et al., 1959). The aqueous mixture was extracted with chloroform (3 × 3 mL). The chloroform layers were washed with water (4 × 5 mL), dried over anhydrous magnesium sulfate, filtered, concentrated, and chromatographed (silica gel, hexanes–ethyl acetate, 4:1 v/v) to give [$^{14}$C]12-MB. The specific activity was determined to be 2.2 Ci/mol; the TLC elution, melting point, and NMR spectrum were identical with that of nonradioactive 12-MB.

*$CF_1$ Preparation.* $CF_1$ was prepared from fresh market spinach as previously described (Lien & Racker, 1971; Binder et al., 1978). In all experiments, purified enzyme having a fluorescence emission ratio 305 nm/340 nm (excitation 280 nm) greater than 1.5 was used. The enzyme was precipitated with an equal volume of saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.2)–1 mM EDTA and stored at 4 °C. Prior to use, $CF_1$ was desalted by passing small volumes of enzyme through two consecutive centrifuge columns (Penefsky, 1977). In some experiments, desalted $CF_1$ was further purified by elution through a Sepharose 4B–anti-ribulosebisphosphate carboxylase column (Cerione et al., 1983). When this was done, essentially complete removal of ribulosebisphosphate carboxylase contaminants was achieved as visualized by SDS–polyacrylamide gel electrophoresis.

Concentrations of $CF_1$ were determined by use of an extinction coefficient of 0.483 mL/(mg·cm) at 277 nm (Bruist & Hammes, 1981) and a molecular weight of 400 000 (Moroney et al., 1983). When volumes were very small and in the presence of ATP, the enzyme concentration was determined with a modification of the Lowry method (Bensadoun, 1976).

Activation of latent $CF_1$ was accomplished by heating $CF_1$ at 63 °C for 4 min in 10 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), 40 mM ATP, and 2 mM EDTA (Moroney et al., 1983). The $Ca^{2+}$-dependent ATPase activity of $CF_1$ was assayed for 5 min at 37 °C in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $CaCl_2$, and approximately 2 $\mu$g/mL enzyme. $P_i$ was determined spectrophotometrically (Taussky & Shorr, 1953). The specific activity of the enzyme was $\geq$25 $\mu$mol mg$^{-1}$ min$^{-1}$.

*Cross-Linking Procedure.* The procedure for cross-linking with 6-MB, 12-MB, and [$^{14}$C]12-MB was as follows. The ammonium sulfate precipitate of $CF_1$ was dissolved in a small volume of buffer A (50 mM Tris–2 mM EDTA, pH 8.0), desalted, and passed through a 3-mL centrifuge column containing Sephadex G-50 equilibrated with cross-linking buffer B (10 mM sodium phosphate, 50 $\mu$M EDTA, and 3 mM ATP, pH 7.0). Stock solutions of cross-linker (50 mM) were made in dimethyl sulfoxide and prepared fresh for each experiment. All incubations prior to photolysis were performed in the dark. The exposed $\gamma$-sulfhydryl ("dark site") on $CF_1$ was labeled by incubation of the enzyme (0.6–2.5 $\mu$M) with a 50-fold excess of cross-linker (6-MB or 12-MB) or *N*-ethylmaleimide (control) for 1 h at room temperature with stirring. The final reaction volume was 1–2 mL, and the final concentration of dimethyl sulfoxide was less than 1.0% (v/v). Unreacted probe was removed by passage of small volumes (<0.5 mL) of the solution through a 3-mL centrifuge column equilibrated with buffer A. To reduce the $\gamma$-disulfide and activate the enzyme, the solution was heated in the dark for 4 min at 63 °C in 10 mM dithiothreitol–40 mM ATP. The excess dithiothreitol was removed by passage of the solution through a centrifuge column equilibrated with cross-linking buffer B, pH 7.0. To react the reduced disulfide with cross-linkers, the enzyme was again incubated with a 50-fold excess of cross-linker for 1 h with stirring. In some experiments, the reduced disulfide was blocked with *N*-ethylmaleimide so that only the dark site was labeled with cross-linker. Two centrifuge columns equilibrated in buffer B, pH 7.0 or 7.8, were used to remove excess probe, and prephotolysis aliquots were removed and prepared for SDS–polyacrylamide gel electrophoresis. Oxygen was removed from the remainder of the enzyme solution by gently bubbling nitrogen through the solution for 10 min prior to photolysis.

Photolysis was carried out for periods of 5–10 min with an Osram 200-W xenon–mercury lamp with a 300-nm Corning glass cutoff filter (>80% transmission at 365 nm). Longer photolysis times did not increase the extent of cross-linking. Samples in test tubes were placed in a holder about 18 cm from the light source. The sample temperature was maintained at 20 °C. After photolysis, aliquots were again removed and prepared for SDS–polyacrylamide gel electrophoresis. The remainder of the enzyme sample was used for determination of the protein concentration and assay of the $Ca^{2+}$-dependent ATPase activity.

A cross-linking experiment designed to test the occurrence of intermolecular cross-links was performed as follows. A solution of $CF_1$ in buffer A was divided into two portions. One portion was labeled with cross-linker as described above on both the dark site and the reduced disulfide. The reserved portion of $CF_1$ was then extensively labeled with fluorescein-maleimide (50 mM stock solution in dimethyl sulfoxide) on $\alpha$, $\beta$, and $\gamma$ subunits by using long reaction times (2 h) and a 100-fold molar excess of probe. Following removal of excess probe with three centrifuge columns, equal volumes of the two solutions of labeled enzyme were combined and photolyzed

as previously described. Aliquots taken both before and after photolysis were prepared for SDS–polyacrylamide gel electrophoresis. Fluorescence was visualized before the gels were stained with a long-wavelength Blak-ray ultraviolet lamp (366 nm).

Cross-linking with 4-maleimidobenzophenone was performed as described above, except acetonitrile was used in preparing stock solutions of cross-linker.

Cross-linking with dimethyl suberimidate was carried out as follows. Stock solutions of cross-linker (50 mM) were prepared in 0.2 M triethanolamine hydrochloride, pH 8.5, which was also the cross-linking buffer. In some experiments, heat-activated enzyme was used, and in some cases, heat activation followed the cross-linking reaction. Incubation periods varied from 30 min to 4 h, and the reaction was stopped by passage of the reaction mixture through a G-50 Sephadex centrifuge column equilibrated with cross-linking buffer to remove excess cross-linker.

A similar procedure was followed for cross-linking with the cleavable reagent dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride, except dithiothreitol was omitted during heat activation. After the incubation period with $CF_1$ and removal of excess cross-linker, 100 mM dithiothreitol was added to the enzyme solution for periods of 15–60 min. Aliquots were removed for analysis both before and after addition of the reducing agent.

Stock solutions of succinimidyl (4-maleimidomethyl)-cyclohexane-1-carboxylate and succinimidyl (4-maleimidophenyl)butyrate were prepared in tetrahydrofuran, and the following cross-linking procedure was followed. For the maleimide reaction, a 50-fold excess of cross-linker was added to $CF_1$ which had been heat activated in the presence of dithiothreitol. After a 1-h incubation period in buffer B, pH 7.0, the pH was raised to 8.0 by passage through a centrifuge column. The reaction was allowed to proceed for 60–90 min, and excess cross-linker was removed with a centrifuge column.

*Polyacrylamide Gel Electrophoresis.* Enzyme samples were put in a solution containing 0.5% (w/v) SDS, 29.0 mM dithiothreitol, 4.0% glycerol, 0.0024% bromophenol blue, and 10 mM sodium phosphate, pH 7.0, boiled for 3 min, and stored at −20 °C. Samples were electrophoresed on SDS–10% (w/v) acrylamide–0.8% (w/v) *N*,*N*′-methylenebis(acrylamide) gels (Chua, 1980). Stacking gels were 4% (w/v) acrylamide–0.4% (w/v) *N*,*N*′-methylenebis(acrylamide). After electrophoresis for 40 min at 80 V and 3.5 h at 105 V, the protein bands were visualized with Coomassie Blue R stain.

*Immunoblotting.* Cross-linked samples of $CF_1$ were prepared and electrophoresed on SDS–polyacrylamide gels as described above. The separated proteins were electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979) in a Bio-Rad Trans-Blot cell at 120 mA for 10 h and then at 210 mA for 2 h in a solution containing 192 mM glycine, 25 mM Tris, 20% (v/v) methanol, and 0.4% SDS at pH 8.3. Following the transfer, the nitrocellulose blots were blocked with 3% bovine serum albumin in 10 mM Tris–0.15 M NaCl, pH 7.5. After 2 h, the filter was washed with the same solution and cut into strips corresponding to gel lanes. Each strip was incubated overnight with 20 μL of previously prepared (Baird & Hammes, 1979) serum containing anti-$\alpha$, -$\beta$, or -$\gamma$ rabbit IgG in 18 mL of solution containing 0.5% bovine serum albumin, 10 mM Tris, and 0.15 M NaCl, pH 7.5. The filters were washed with this solution and incubated for 2 h with horseradish peroxidase conjugated goat anti-rabbit IgG. The filters were again washed extensively and developed with 0.015% 4-chloro-1-naphthol, 8 mM Tris, 0.12 M NaCl, 20% (v/v) methanol, and 0.01% $H_2O_2$ for 2 min. Filters were washed with water and photographed the same day.

*Analysis of Polyacrylamide Gels.* The extent of cross-linking was determined in two ways. The relative amount of protein in each of the bands in the SDS–polyacrylamide gels was measured by determination of the Coomassie Blue staining intensity at 580 nm with an ISCO gel scanner. To determine the range for which the protein concentration is proportional to the Coomassie Blue absorbance, standard curves were generated for each polypeptide with enzyme that was not cross-linked. Densitometry scans were xeroxed, and peaks were cut out and weighed. When $CF_1$ was cross-linked with [$^{14}$C]12-MB, the destained polyacrylamide gel lanes were also sliced into 2-mm pieces. The amount of radioactivity in each slice was determined by dissolving the gel in 0.5 mL of 30% hydrogen peroxide at 90 °C for 2 h followed by addition of 200 units of catalase to destroy the excess peroxide (Anderson & Hammes, 1985).

In an attempt to determine the molecular weight of the major cross-linked species, cross-linked samples of $CF_1$ and six molecular weight standards were run on five different concentrations of polyacrylamide (5–9%). For each sample, a plot of −log relative migration vs. percent acrylamide was constructed. This analysis is based on the relationship (Ferguson, 1964):

$$\log R_f = \log M_0 - K_R C$$

where $R_f$ is the relative electrophoretic mobility in a gel of concentration $C$, $M_0$ is the electrophoretic mobility at zero gel concentration (a constant for standard SDS binding proteins), and $K_R$ is the retardation coefficient. log $M_R$, where $M_R$ refers to molecular weight, is a linear function of $K_R$ for standard proteins. A plot of log $M_R$ vs. $K_R$ was constructed for the molecular weight standards and used to estimate the molecular weights of the unknown species.

In addition to this two-plot analysis, the empirically observed linear relationship between $R_f$ and log $M_R$ was used to estimate the molecular weight of the cross-linked species (Weber & Osborn, 1969). Separate plots of $R_f$ vs. log $M_R$ were constructed for each gel concentration employed.

## Results

The chemical cross-linkers used were designed to cross-link specifically the $\gamma$-polypeptide of $CF_1$ to the larger polypeptides, $\alpha$ and $\beta$. A maleimide adduct was desired because the $\gamma$-polypeptide has a reactive cysteine ("dark-site sulfhydryl") as well as a disulfide bond that gives two reactive sulfhydryls when reduced. In this and previous work (Cantley & Hammes, 1976), it was shown that incubation of $CF_1$ with *N*-[$^3$H]ethylmaleimide results in incorporation of >90% of the radioactivity into the $\gamma$-polypeptide. Furthermore, the specific activity of $CF_1$ is unchanged after labeling with *N*-ethylmaleimide. In addition to being specific for the $\gamma$-polypeptide, the cross-linkers are long enough (>10 Å) to permit some flexibility in the enzyme structure. An important factor for this study is that the chemical modification must have little or no effect on the specific activity of the enzyme. In addition, to assure that only intramolecular cross-linking occurred, the total protein concentration employed in each experiment was kept low (0.6–2.5 μM). None of the results obtained was found to be dependent on the protein concentration over this range. An additional experiment designed to detect intermolecular cross-links was performed. $CF_1$ which had been extensively labeled with fluoresceinmaleimide was added to an equal volume and concentration of $CF_1$ labeled with cross-linker on all three $\gamma$-sulfhydryls. Samples were then

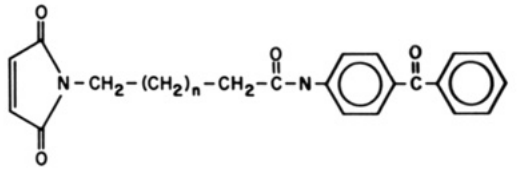

FIGURE 1: Structures of the heterobifunctional photoaffinity cross-linking reagents synthesized and used in cross-linking studies with $CF_1$: $n = 3$, 6-MB; $n = 9$, 12-MB.

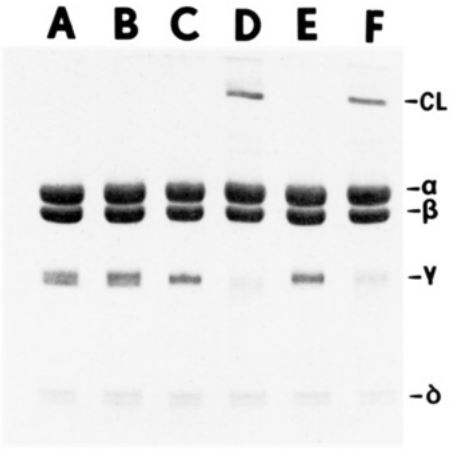


FIGURE 2: Photograph of a 10% SDS–polyacrylamide gel after electrophoresis demonstrating the effect of cross-linking of $CF_1$ with 6-MB and 12-MB. $CF_1$ reacted with the following: *N*-ethylmaleimide, prephotolysis (A) and postphotolysis (B); 6-MB, prephotolysis (C) and postphotolysis (D); and 12-MB, prephotolysis (E) and postphotolysis (F). The ε-polypeptide runs with the dye front on this gel.

photolyzed and subjected to SDS–polyacrylamide gel electrophoresis. Visualization of the fluorescence before staining indicated that the samples were fluorescently labeled on the α-, β-, and γ-polypeptides; however, fluorescence was absent in the bands corresponding to cross-linked polypeptides. Thus, these bands are a result of intramolecular cross-linking between polypeptides of nonfluorescently labeled $CF_1$ only.

Preliminary studies with the cross-linking reagents dimethyl suberimidate, dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride, succinimidyl (4-maleimidomethyl)cyclohexane-1-carboxylate, and succinimidyl (4-maleimidophenyl)butyrate, all of which involve reaction with amino groups on one or both ends, showed that these reagents cause major losses in $CF_1$ ATPase activity (60–100% activity loss). Furthermore, cleavage of the cross-link formed by dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride did not restore ATPase activity significantly. Use of the heterobifunctional photoaffinity cross-linker 4-maleimidobenzophenone was more encouraging. Cross-linking was demonstrated, and only a 25–30% loss in activity was observed. The short bridge between the reactive ends of the reagent, however, probably contributed to the observed activity loss.

The structures of the longer photoaffinity cross-linkers that were synthesized, 6-MB and 12-MB, are shown in Figure 1. The SDS–polyacrylamide gel in Figure 2 illustrates typical results of cross-linking experiments with 6-MB (20-Å bridge) and 12-MB (30-Å bridge). Photolysis at pH 7.8 and the resulting cross-linking of the enzyme resulted in a 12% (one experiment) and 7% ± 6% (average of seven experiments) loss in specific activity when the 6- and 12-carbon cross-linkers, respectively, were employed. Photolysis at pH 7.0 resulted in slightly larger losses in ATPase activity: decreases of 20% ± 4% (6-MB, average of four experiments) and 11% ± 4% (12-MB, average of two experiments) were obtained. If the enzyme is activated by a 3-h room temperature incubation in

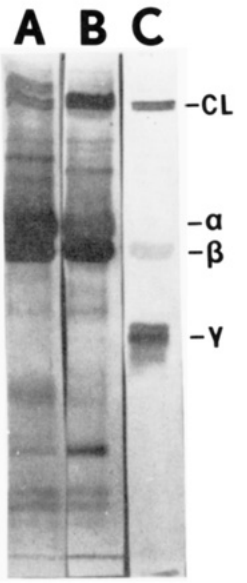


FIGURE 3: Immunoblotting analysis of cross-linked $CF_1$. $CF_1$ was cross-linked with 12-MB, electrophoresed on a 10% SDS–polyacrylamide gel, and electrophoretically transferred to nitrocellulose. Filter strips were incubated with rabbit IgG antibody as follows: anti-α (A), anti-β (B), and anti-γ (C). Visualization of protein bands was accomplished by incubation of filters with horseradish peroxidase conjugated goat anti-rabbit IgG and development with 4-chloro-1-naphthol.

50 mM Tris-HCl, 1 mM EDTA, 50 mM dithiothreitol, and 3 mM ATP at pH 8.0, rather than 4 min at 63 °C, no loss in activity occurs after photolysis at pH 7.8 with 12-MB (one experiment) or 6-MB (two experiments). Reaction of the maleimide portion of the reagents with $CF_1$ prior to photolysis did not cause any significant loss in the enzymatic activity of $CF_1$. As a control, an enzyme sample was labeled with *N*-ethylmaleimide (gel lane A) and then photolyzed (gel lane B). The specific ATPase activity of the control sample was unchanged after photolysis. When *N*-[$^3$H]ethylmaleimide was reacted with enzyme under identical conditions, ~2.9 molecules of the reagent were bound per enzyme molecule. The stoichiometry of cross-linkers bound to the enzyme, determined by using [$^{14}$C]12-MB, was 2.0 molecules per $CF_1$ molecule. Examination of the polyacrylamide gel shown in Figure 2 indicates that a large percentage of the γ-polypeptide is removed from its normal position in the gel after photolysis (lanes C–F). New high molecular weight bands (CL) also appear in these samples. The band associated with the γ-polypeptide is somewhat broadened after photolysis, probably due to cross-linking within the γ-polypeptide. Three additional methods were employed to characterize the polypeptide chain composition of the cross-linked bands and to quantify the extent of loss of protein in the bands associated with the α-, β-, and γ-polypeptides.

The results of an immunoblotting analysis performed after cross-linking $CF_1$ with 12-MB are shown in Figure 3. The anti-γ antibody has a small degree of cross-reactivity with the β-polypeptide (C); however, the intensity of the γ and cross-linked bands and the results of probing with anti-α (A) and anti-B (B) antibodies indicate βγ and/or αγ cross-linking has occurred. Similar results (not shown) were obtained from immunoblotting experiments performed after cross-linking with 6-MB.

The stain intensity on SDS–polyacrylamide gels was measured before and after photolysis under conditions where the protein concentration is proportional to the absorbance of Coomassie Blue. Some typical scans of polyacrylamide gels

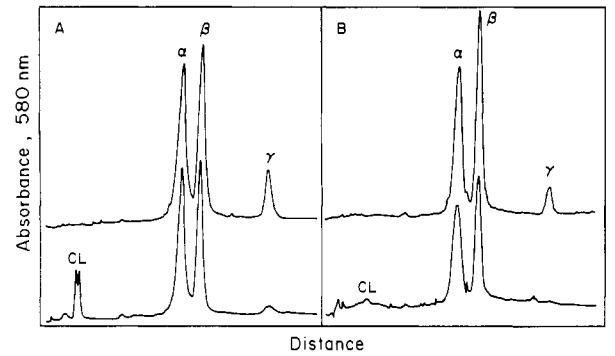


FIGURE 4: Spectrophotometric traces at 580 nm of a Coomassie Blue stained SDS–polyacrylamide gel before (upper scans) and after (lower scans) cross-linking of $CF_1$ with 12-MB. Scans are shown for 6-$\mu$g (A) and 3-$\mu$g (B) protein samples to demonstrate the appearance of cross-linked protein, CL, and the disappearance of the $\gamma$-polypeptide (lower left) and of $\alpha$- and $\beta$-polypeptides (lower right) from their normal positions following photolysis. The absorbance scale in (B) is expanded about 2-fold relative to that used for (A).

before and after the cross-linking of $CF_1$ with 12-MB are shown in Figure 4. The two sets of scans (A and B) correspond to 6 and 3 $\mu$g of enzyme, respectively; the absorbance range is expanded approximately 2-fold in (B) relative to (A). The peak corresponding to the cross-linked species (CL) appears following photolysis as shown in the lower scan of (A). The removal of the $\gamma$-polypeptide from its normal position is also clearly demonstrated in this pair of scans. A small percentage of protein (<5%) remained in the stacking gel following photolysis. The scans in (B) serve to demonstrate the cross-linking of $\alpha$- and $\beta$-polypeptides after photolysis. In this case, however, the low enzyme concentration prevents the clear detection of cross-linked bands. Because results based on Coomassie Blue protein staining are not always reliable (Leffak, 1983), a second method of quantitation was employed. [$^{14}$C]12-MB was used to cross-link $CF_1$, and pre- and postphotolysis aliquots were subjected to SDS–polyacrylamide gel electrophoresis. The amount of radioactivity incorporated into each section of gel was determined. Results of these experiments indicate that ~70% of the label is present on the $\gamma$-polypeptide before photolysis and that ~50% of this radioactivity is removed from $\gamma$ following photolysis. Furthermore, in postphotolysis samples, >50% of the radioactivity appears in cross-linked bands above the $\alpha$-polypeptide. Radioactivity is also present in the stacking gel of postphotolysis samples, but the total amount of radioactivity in the resolving gel is essentially the same for pre- and postphotolysis samples (<±5%). A summary of the extent of cross-linking of $\alpha$-, $\beta$-, and $\gamma$-polypeptides with 12-MB and [$^{14}$C]12-MB is given in Table I. Similar results were obtained with 6-MB: 69% loss of $\gamma$-polypeptide (by densitometry) at pH 7.0. These results correlate well with the immunoblotting analysis and indicate that more $\beta\gamma$ than $\alpha\gamma$ cross-links are formed. Futhermore, more $\gamma$-polypeptide is removed (~48–73%) compared to removal of the larger polypeptides (~30–37% total $\alpha + \beta$ removal). Since $CF_1$ has three $\alpha$- and three $\beta$-polypeptides and only one $\gamma$-polypeptide (Moroney et al., 1983), this result is not unexpected.

In an attempt to determine the precise stoichiometry of the cross-linked polypeptides, a Ferguson gel analysis was performed (Ferguson, 1964). While SDS–polyacrylamide gel electrophoresis is a reliable technique for determining the molecular weights of many proteins, the introduction of artificial chemical cross-links has been shown to restrict the binding of SDS and alter electrophoretic mobilities (Davies & Stark, 1970). A comparison of electrophoretic mobilities

Table I: Effect of 12-MB Cross-Linking on $CF_1$

| method | % decrease after photolysis $\alpha$ | $\beta$ | $\gamma$ | % activity loss | no. of expt |
|---|---|---|---|---|---|
| densitometry | | | | | |
| pH 7.8 | 11 ± 8 | 19 ± 6 | 49 ± 5 | 7 ± 6[a] | 3 |
| pH 7.0 | 17 ± 7 | 20 ± 13 | 73 ± 1 | 11 ± 4 | 2 |
| radioactivity | | | | | |
| pH 7.8 | | | 50 ± 3 | 7 ± 6[a] | 2 |

[a] Average of seven experiments.

at a variety of gel concentrations is a method often used to detect anomalously migrating proteins (Neville, 1971). The results of such an analysis indicate that the cross-linked species have a free electrophoretic mobility greater than twice the value obtained for the standard SDS binding proteins, suggesting that they do indeed migrate anomalously and, therefore, no molecular weight assignment can be made on the basis of electrophoretic migration. When simple plots of $R_f$ vs. log $M_R$ were constructed and compared to the migration of standard proteins, the molecular weights of the major cross-linked species were estimated to range from 105 000 (5% gel) to 160 000 (10% gel). The lower limit suggests formation of $\alpha\gamma$ (molecular weight 96 000) or $\beta\gamma$ (molecular weight 93 000) and the upper limit $\alpha_2\gamma$, $\beta_2\gamma$, or $\alpha\beta\gamma$ (molecular weight 149 000–155 000) formation. (This assumes one $\gamma$-polypeptide per $CF_1$ molecule.) The extent of cross-linking and the amount of Coomassie Blue stain in the CL bands do not appear to support formation of the latter species. Further evidence that argues in favor of a 1:1 stoichiometry was obtained by labeling $CF_1$ with cross-linker on the $\gamma$ "dark site" only. One high molecular weight band appears following photolysis in the same position as the upper band of the doublet seen when all three sulfhydryl sites are labeled. Since only one $\gamma$-sulfhydryl site is initially labeled with cross-linker, the new species must be either $\alpha\gamma$ or $\beta\gamma$.

To be certain that trace amounts of ribulosebisphosphate carboxylase were not influencing the results, some experiments were done with $CF_1$ that had been treated with a ribulosebisphosphate carboxylase antibody affinity column. Cross-linking experiments with $CF_1$ treated in this way gave results identical with those reported above. No significant cross-linking to the $\delta$- and $\epsilon$-polypeptides was observed, although a small depletion of $\epsilon$- from its normal position in the polyacrylamide gel may occur. A band corresponding to $\gamma\epsilon$ was not observed, so that any cross-linked $\epsilon$ must be part of high molecular weight aggregates.

## Discussion

Chemical cross-linking studies have been performed previously with $CF_1$ (Baird & Hammes, 1976), and recently similar studies were carried out to determine the organization of the polypeptide chains of the entire chloroplast ATP synthase (Suss, 1986). Chemical modifications performed in these previous studies, as well as preliminary work in the present study with $CF_1$, indicate that complete or near-complete loss in activity results with reagents primarily targeted at amino groups. The difficulties in maintaining enzymatic activity during extensive intrasubunit cross-linking are obvious. In an attempt to minimize the loss in specific ATPase activity caused by amino group modifications and to gain information about the mechanism of catalysis of $CF_1$, two new cross-linking reagents, 6-MB and 12-MB, were synthesized. These reagents were designed to form specific cross-links between the $\gamma$-polypeptide and the $\alpha$- and $\beta$-polypeptides. The $\gamma$-specific maleimide moiety, the nonspecific photoactivated benzophenone functional group, and the length of these cross-linkers (20 and

30 Å) were features that contributed to their usefulness.

The goal of this study was to test the suggestion that a rotation of the $\alpha$–$\beta$-polypeptides about the $\gamma\delta\epsilon$-polypeptides occurs during the normal catalytic cycle of $CF_1$. To date, no direct evidence for or against this mechanism has been found. The results presented in this work (extensive $\alpha\gamma$ and/or $\beta\gamma$ cross-linking with ~7% activity loss with 12-MB and ~12% activity loss with 6-MB) indicate that polypeptide rotation is not essential for catalysis.

While immunoblotting analyses, spectrophotometric scans of Coomassie Blue stained SDS–polyacrylamide gels, and radioactive gel analyses show removal of $\alpha$, $\beta$, and $\gamma$ from their normal positions on the gels and indicate ~50% of $\gamma$ is cross-linked, unambiguous identification of the constituent polypeptides of the cross-linked products is complicated by anomalous migration of high molecular weight cross-linked species and the qualitative nature of immunoblotting. However, the results reported suggest that the cross-linked species corresponds to $\beta\gamma$ and/or $\alpha\gamma$.

In summary, the results obtained through the use of new heterobifunctional photoaffinity cross-linking reagents suggest that large movements of the $\alpha$–$\beta$-polypeptides with respect to the smaller $\gamma$-, $\delta$-, and $\epsilon$-polypeptides are not required during the catalytic cycle of $CF_1$. However, this maintenance of overall structural asymmetry during catalysis does not exclude the possibility of functionally equivalent catalytic sites.

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