Selective Modification of an α Subunit of Chloroplast Coupling Factor 1†

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ABSTRACT: Lucifer yellow (4-amino-*N*-[3-(vinylsulfonyl)phenyl]naphthalimide-3,6-disulfonate), a fluorescent probe that can react covalently with sulfhydryl or amino groups, has been used to modify chloroplast coupling factor 1 (CF_1). Conditions are described under which Lucifer yellow selectively labels the α subunit of CF_1 to the extent of about 1 mol of probe per mole of CF_1. An especially reactive amino group is apparently labeled, and modification has little effect on the ATPase activity of the enzyme. Lucifer yellow is a useful probe for fluorescence energy transfer measurements. The distances between this probe and fluorescent and absorbing molecules attached to seven specific sites on the β, γ, and ϵ subunits were determined. These distances converge to a single location. In addition to providing further information about the structure of CF_1, these results suggest that the α subunits of CF_1 are not structurally equivalent.

Chloroplast coupling factor 1 (CF_1)[1] catalyzes ATP synthesis by using the energy of the transmembrane proton gradient. Its structure and mechanism are similar to those of the coupling factors from mitochondria and bacteria. CF_1 consists of five distinct subunits (α, β, γ, δ, and ϵ) having molecular weights ranging from 59 000 to 15 000 and a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (McCarty & Moroney, 1984). The α and β subunits are the site of nucleotide binding and catalysis. While some information about the overall structure of the coupling factors is available, little is known about the organization of subunits. X-ray crystallography of the mitochondrial enzyme has not, as yet, been able to distinguish unique domains that correspond to specific subunits (Amzel et al., 1982), and CF_1 crystals of sufficient size and uniformity for X-ray analysis have not been prepared. In contrast, measurements of steady-state fluorescence measurements from probes attached to specific sites on CF_1 have provided information about the structure of the enzyme in solution (Cantley & Hammes, 1976; Cerione & Hammes, 1982; Nalin et al., 1983; Nalin & McCarty, 1984) and in a reconstituted complex (Cerione et al., 1983; Snyder & Hammes, 1984).

Energy transfer between these fluorescent probes attached to the protein permits the calculation of distances between specific sites on the enzyme. CF_1 contains several sulfhydryl residues that can be distinguished by their reactivity with maleimides. The sulfhydryl residue of the γ subunit of CF_1 bound to thylakoids that reacts with *N*-ethylmaleimide only in the light is located in a hydrophobic domain and is inaccessible to the medium under deenergized conditions or when enzyme is solubilized (McCarty et al., 1972; McCarty & Fagan, 1973; Nalin et al., 1982). A second sulfhydryl of γ in membrane-bound and soluble CF_1 can be readily modified with maleimides. CF_1 also contains a single disulfide bond in the γ subunit (Nalin & McCarty, 1984) which is likely to be involved in activation of the coupling factor for both ATP synthesis and hydrolysis (Arana & Vallejos, 1982; Ketcham et al., 1984). Reduction of the disulfide activates the ATPase of the soluble enzyme (Arana & Vallejos, 1982; Nalin & McCarty, 1984). Fluorescence energy transfer measurements between probes bound to the various sulfhydryl residues of the γ subunit and nucleotide analogues bound to the three nucleotide sites of CF_1 (Cerione & Hammes, 1982; Snyder & Hammes, 1984, 1985) have begun to provide a three-dimensional view of the organization of the nucleotide binding sites on the α/β subunits and their relationship to the γ subunit.

The use of steady-state fluorescence and resonance energy transfer measurements to study the structure of CF_1 requires specific labeling of unique sites in the protein. While labeling of the β, γ, and ϵ subunits has been possible, specific modification of the α subunits has not previously been reported. The single sulfhydryl residue in α (Deno et al., 1983) is inaccessible to modification in both membrane-bound and soluble CF_1. In this paper, we report on a specific modification of an α subunit of CF_1 with 4-amino-*N*-[3-(vinylsulfonyl)-phenyl]naphthalimide-3,6-disulfonate (Lucifer yellow). This fluorescent probe can label either sulfhydryl residues or amino groups. It has a high quantum yield, is environmentally sensitive, and can be used as either a donor or an acceptor for fluorescence energy transfer measurements. We show that Lucifer yellow reacts with a single amino group of the α subunit of soluble CF_1. In addition, fluorescence energy transfer measurements between the bound Lucifer yellow and probes attached to other sites on CF_1 indicate that Lucifer yellow modifies a single, structurally unique α subunit.

Materials and Methods

Preparation of CF_1. CF_1 was prepared from market spinach by a combination of the methods described by Binder et al. (1978) and Lien & Racker (1971). After elution of the enzyme from DEAE-cellulose, further purification was achieved by chromatography on DEAE-Sephadex. An additional wash of the thylakoids with 10 mM NaCl was used to remove contaminating ribulosebisphosphate carboxylase prior to extraction of CF_1 with EDTA (Moroney & McCarty, 1982).

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; SDS, sodium dodecyl sulfate; DMSM, *N*-(2,5-dimethoxystilben-4-yl)maleimide; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine 5′-triphosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DCCD, dicyclohexylcarbodiimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tricine, *N*-[(tris(hydroxymethyl)methyl]glycine.

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The enzyme was greater than 95% pure as judged by SDS–polyacrylamide gel electrophoresis. CF_1 was stored as an ammonium sulfate precipitate at 4 °C. Prior to use, CF_1 was desalted on a 1 × 10 cm column of Sephadex G-50. For small volumes, centrifuge columns (Penefsky, 1977) were used. The molar concentration of CF_1 was determined by a modification of the Lowry method (Peterson, 1977) or from the absorbance of the protein solution at 277 nm using an extinction coefficient of 0.483 $mL \cdot mg^{-1} \cdot cm^{-1}$ (Bruist & Hammes, 1982) and a molecular weight of 400 000 (Moroney et al., 1983). Spinach thylakoids (McCarty & Racker, 1967), CF_1-depleted thylakoids (Nelson & Eytan, 1979), and DCCD-sensitive ATPase (Pick & Racker, 1979; Cerione & Hammes, 1982) were prepared as described.

Covalent Modification of CF_1. Modification of CF_1 with Lucifer yellow was routinely performed in 50 mM Bicine–NaOH (pH 9.0) and 3 mM $MgCl_2$. CF_1 was incubated at 1–5 mg/mL with 50–60 μM Lucifer yellow for 15 min at room temperature. Dithiothreitol was added to a final concentration of 1 mM, and the modified enzyme was separated from the unbound Lucifer yellow by passage either through two successive centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA or through 1 × 10 cm columns of Sephadex G-50. The stoichiometry of Lucifer yellow labeling was determined by using an extinction coefficient of 1.22×10^4 $M^{-1} \cdot cm^{-1}$ at 428 nm (Stewart, 1981). The modified enzyme remained stable at 4 °C as an ammonium sulfate precipitate for several weeks.

Selective modifications of the sulfhydryl residues of the γ subunit of soluble CF_1 were performed essentially as described previously (Nalin & McCarty, 1984). The stoichiometry of DMSM labeling was calculated by using an extinction coefficient of 1.40×10^4 $M^{-1} \cdot cm^{-1}$ at 344 nm (Snyder & Hammes, 1984). When both probes were present, the absorbance was corrected for bound Lucifer yellow to obtain the DMSM stoichiometry. For modification of the accessible sulfhydryl on the γ subunit, the ε subunit of CF_1 was first removed by ethanol extraction of CF_1 bound to a DEAE-cellulose column (Richter et al., 1984). $CF_1(-\epsilon)$ was incubated at 2–4 mg/mL with 50 μM DMSM in 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA for 5–10 min at room temperature. Dithiothreitol was added to a final concentration of 1 mM, and unbound DMSM was removed by column centrifugation. Following modification and removal of unbound maleimide, ε subunit was reconstituted (Richter et al., 1984). Preferential modification of the sulfhydryl on the γ subunit that reacts only in energized thylakoids was done by using thylakoids prelabeled with 2 mM *N*-ethylmaleimide for 20 min in the dark followed by centrifugation to remove the unbound *N*-ethylmaleimide. The light-accessible sulfhydryl was labeled during illumination (2.5×10^6 $ergs \cdot cm^{-2} \cdot s^{-1}$ of white light) of the thylakoids at 50 μg of $chlorophyll \cdot mL^{-1}$ for 2 min at room temperature in 50 mM Tricine–NaOH (pH 8.0), 50 mM NaCl, and 5 mM $MgCl_2$ in the presence of 55 μM DMSM. CF_1 was then isolated (Cerione et al., 1983). To label the sulfhydryl residues that form the disulfide bond in γ, CF_1 at 3 mg/mL was first incubated with 3 mM *N*-ethylmaleimide for 20 min at room temperature to block the accessible sulfhydryls, followed by treatment with 55 mM dithiothreitol for 90 min at room temperature to reduce the disulfide bond. Dithiothreitol was removed by two successive column centrifugations, and the sulfhydryl residues were modified by incubation of the reduced enzyme with 50 μM DMSM for 10 min followed by addition of 2 mM *N*-ethylmaleimide. For all cases, SDS–polacrylamide gel electrophoresis of the modified CF_1 samples showed a specific incorporation of DMSM into the γ subunit.

Specific modification of the ε subunit was done after isolation of ε by the method of Richter et al. (1984). Isolated ε subunit at about 0.2 mg/mL was incubated with 30 μM DMSM in 25 mM Tris-HCl (pH 8.0), 2 mM ATP, 30% glycerol, and 20% ethanol for 30 min. Dithiothreitol was added to the mixture, and modified ε was then reconstituted with $CF_1(-\epsilon)$ by incubating for 10 min in 25 mM Tris-HCl (pH 8.0). Following chromatography on Sephadex G-50 to remove unbound ε and DMSM, the reconstituted enzyme was then labeled with Lucifer yellow as described above.

Nucleotide Binding to CF_1. The nucleotide binding sites of CF_1 have been characterized on the basis of their specificity and sensitivity to medium nucleotide and metal ions (Bruist & Hammes, 1981). These sites are located on β subunits or at the interface of α and β subunits (Bruist & Hammes, 1981; Kambouris & Hammes, 1985). Site 1 is the exchangeable nucleotide site that binds ATP or ADP in the presence or absence of metal ion and retains the bound nucleotide when medium nucleotide is removed. Site 2 binds MgATP with a very high affinity, and the MgATP does not exchange with medium nucleotide. Site 3 has the weakest binding affinity for both ADP and ATP of the three sites characterized and is thought to be a catalytic site.

Specific labeling of the ADP-exchangeable site (site 1) was obtained by incubating Lucifer yellow labeled CF_1 (2.5 mg/mL) with 50 μM TNP-ATP for 2–3 h in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA followed by two column centrifugations to remove unbound and dissociable nucleotides from CF_1. The stoichiometry of labeling was determined from the absorbance of TNP-ATP by using an extinction coefficient of 2.51×10^4 $M^{-1} \cdot cm^{-1}$ at 418 nm for the bound nucleotide (Cerione & Hammes, 1982). Stoichiometries of 0.85–1.0 mol of TNP-ATP/mol of CF_1 were obtained for binding at site 1 after correction for light scattering and the absorbance of Lucifer yellow at 418 nm. Site 1 and site 2 (MgATP site) were labeled together by incubating CF_1 (3 mg/mL) with 0.1 mM TNP-ATP in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 6 mM $MgCl_2$ for 3 h. Unbound TNP-ATP was removed by two successive column centrifugations. The stoichiometry of labeling was 1.9–2.0 mol of TNP-ATP/mol of CF_1. Similarly, site 2 could be blocked by prelabeling CF_1 with MgATP prior to labeling of site 1 with TNP-ATP. The stoichiometry of bound nucleotide for this sample was 1.0 mol of TNP-ATP per mole of CF_1. Site 3 labeling was performed by tritrating the Lucifer yellow labeled CF_1 with 0.2–20 μM TNP-ATP (Snyder & Hammes, 1984).

Spectrophotometric Measurements. Absorption spectra were determined with either a Beckman DU-7 or a Cary 118 spectrophotometer. Absorbances were corrected for light scattering and for spectral overlaps of the probes. Fluorescence measurements were made on a Hitachi Perkin-Elmer MPF-44B spectrofluorometer equipped with corrected spectrum and polarization accessories. The steady-state polarizations were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator (Cerione & Hammes, 1982). Quantum yields of DMSM and Lucifer yellow fluorescence were determined by the ratio method (Parker & Reese, 1966). Sodium fluorescein in 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA and quinine sulfate in 0.1 N H_2SO_4 were used as standards and were assumed to have quantum yields of 0.92 (Weber & Teale, 1957) and 0.70 (Scott et al., 1970), respectively.

Fluorescence Resonance Energy Transfer Measurements. Fluorescence resonance energy transfer measurements were

performed as described previously (Snyder & Hammes, 1984). For all measurements, donor labeling of CF_1 was done prior to acceptor labeling. Corrections were made for acceptor stoichiometry, probe absorbance, and light scattering. Distances determined from energy transfer efficiencies were calculated by using

$$E = \sum_{i=1}^{N_A}(R_0/R_i)^6/[1 + \sum_{i=1}^{N_A}(R_0/R_i)^6] \quad (1)$$

where N_A is the number of acceptors, R_i is the distance between the ith donor–acceptor pair, and R_0 is the distance when the transfer efficiency is 0.5 for a single donor–acceptor pair. Equation 1 assumes each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of enzyme. R_0 can be determined from (Förster, 1959)

$$R_0 = (9.79 \times 10^3)(\kappa^2 J Q_0 n^{-4})^{1/6} \quad (2)$$

where n is the refractive index of the medium, J is the spectral overlap integral, and κ^2 is the orientation factor for dipolar coupling between donors and acceptors (Dale & Eisinger, 1974). In this study, κ^2 was assumed to be $^2/_3$, the case in which the rotation of the donor and acceptor moieties is rapid relative to the lifetime of the donor fluorescence. The probable uncertainty in the measured distances due to this assumption has been discussed elsewhere [cf. Snyder & Hammes, (1985)].

Tryptic Digestion and Peptide Mapping of Labeled CF_1. CF_1 labeled with Lucifer yellow and control CF_1 (4 mg/mL) were dissolved in 0.5 mL of 0.2 M *N*-ethylmorpholine–acetate (pH 8.0) and placed in a boiling water bath for 30 s. After the samples were cooled to room temperature, 50 μg of trypsin in 1 mM H_2SO_4 was added, and the digestions were allowed to incubate at 37 °C overnight. A second addition of trypsin was made, and the samples were incubated until clear. Following lyophilization, aliquots containing 150–200 μg of protein in 20 μL of 0.2 M *N*-ethylmorpholine–acetate buffer were injected into the HPLC. The HPLC system consisted of a Beckman Model 342 gradient liquid chromatograph with a single wavelength detector set at 254 or 214 nm. Separations were performed on an Altex Ultrasphere ODS column (5 μm, 0.5 × 25 cm) using a linear gradient of 0.1% phosphoric acid (solvent A) and 0.1% phosphoric acid in acetonitrile (solvent B) from 2% to 60% solvent B at 2.0 mL/min over 90 min (Moroney et al., 1984).

Chemicals. Lucifer yellow and quinine sulfate were obtained from Aldrich Chemical Co. DMSM and TNP-ATP were from Molecular Probes, Inc. Disodium fluorescein was obtained from Eastman Chemical Co. Acetonitrile (HPLC grade) was purchased from Burdick and Jackson Laboratories, Inc. All other chemicals and reagents were of highest quality grade. Deionized, distilled water was used for all reagents and for HPLC.

Results

Lucifer Yellow Modification of Isolated CF_1. Lucifer yellow VS is a vinyl sulfone derivative of naphthylimide that covalently reacts with amino and sulfhydryl groups (Stewart, 1981). Originally synthesized for use in marking nerve cells (Stewart, 1978), its fluorescence properties make it suitable as an extrinsic probe for protein fluorescence studies. Its relatively high quantum yield, long wavelength excitation, and stability make it ideally suited for fluorescence energy transfer measurements as either a donor or an acceptor.

In solution, the fluorescence of Lucifer yellow is sensitive to the polarity of the solvent, exhibiting both a blue shift in the emission peak and an increase in the quantum yield as the polarity of the solvent decreases (Table I). As with other naphthylimides (Stewart, 1978), the fluorescence of Lucifer yellow is insensitive to H^+ concentration in the range of pH 5.5 to pH 10.0 (not shown). When reacted with soluble CF_1, the fluorescence emission peak is similar to that of the free dye in ethanol. Together with the high degree of fluorescence polarization of Lucifer yellow bound to CF_1 (0.30), these results suggest that the probe is bound in a hydrophobic environment on the protein with restricted rotational freedom. Incubation of CF_1 with Lucifer yellow in the presence of dithiothreitol or preincubation of Lucifer yellow with dithiothreitol destroys the reactivity of the probe.

Table I: Fluorescence of Lucifer Yellow[a]

solvent[b]	dielectric constant	emission max (nm)	quantum yield[d]	polarization
H_2O	78.6	547	0.35	0.02
ethylene glycol	37	543	0.53	nd
ethanol	24.3	535	0.82	nd
CF_1 bound[c]	nd[e]	537	0.36	0.30

[a] Corrected steady-state fluorescence of Lucifer yellow was measured in a Hitachi Perkin-Elmer spectrofluorometer using 428-nm excitation. [b] The concentration of Lucifer yellow was 25 μM in each solvent. [c] Lucifer yellow modification of CF_1 was performed as described under Materials and Methods. Fluorescence of Lucifer yellow labeled CF_1 at 1.8 mg/mL was measured in 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA. [d] Disodium fluorescein was used as a standard. [e] nd, not determined.

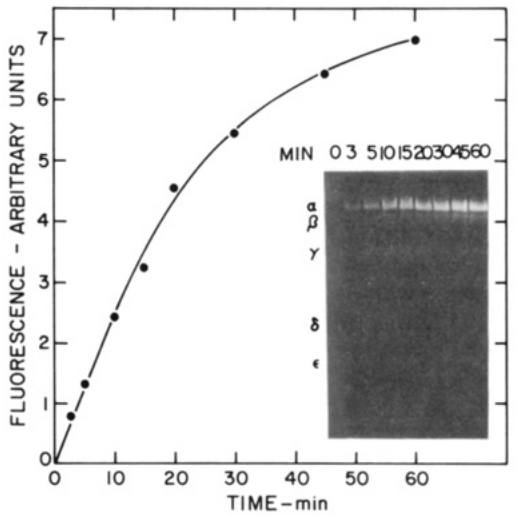

FIGURE 1: Time course of Lucifer yellow labeling of isolated CF_1. Soluble CF_1 at 2.0 mg/mL in 50 mM Bicine–NaOH (pH 9.0) and 3 mM $MgCl_2$ was incubated with 50 μM Lucifer yellow. At the times indicated, 50-μL aliquots were diluted into 150 μL of 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA containing 25 mM dithiothreitol and the samples passed through a 1 × 10 cm column of Sephadex G-50 to remove unbound Lucifer yellow. Fluorescence of bound Lucifer yellow was measured at 535 nm with 430-nm exiciting light. Inset: SDS–polyacrylamide gel electrophoresis of 20 μg of CF_1 of samples from another, identical experiment. Gel was photographed under UV light with Polaroid type 57 film (high speed) using filters to collect light between 490 and 550 nm.

The labeling of CF_1 (5 μM) with 50 μM Lucifer yellow at pH 9.0 is relatively rapid for the first 20 min and becomes much slower thereafter (Figure 1). During the rapid phase of the reaction, the probe is incorporated almost entirely into the α subunit. With longer incubation times, the extent of labeling of the β, γ, and ε subunits slowly increased. In all subsequent experiments, therefore, CF_1 was incubated with 50–60 μM Lucifer yellow for 15 min at pH 9.0 in the presence of 3 mM $MgCl_2$. Mg^{2+} enhanced the rate of Lucifer yellow incorporation by 2-fold but did not affect the extent of labeling (not shown). In each case, the probe was incorporated nearly entirely into the α subunit. The extent of incorporation varied

Table II: Fluorescence Energy Transfer Measurements

site	donor	acceptor	quantum yield of donor	polarization of donor	R_0[a]	E[b]	distance (Å)
SH, dark	DMSH	LY	0.13	0.36	26.3	0.31	30
SH, light	DMSM	LY	0.09	0.42	26.6	0.10	38
S–S	DMSM	LY	0.11	0.40	27.4	<0.05	>43
AdN[c] site 1	LY[d]	TNP-ATP	0.36	0.30	34.8	<0.05	>57
AdN site 2	LY	TNP-ATP	0.36	0.30	34.8	0.43	36
AdN site 3	LY	TNP-ATP	0.36	0.30	34.8	0.41	37
ϵ-SH	DMSM	LY	0.26	nd[e]	31.8	0.16	42

[a] R_0 calculated by using eq 2 and the spectral properties of the donor and acceptor molecules. [b] Efficiency per mole of bound acceptor. [c] AdN, adenine nucleotide. [d] LY, Lucifer yellow. [e] nd, not determined.

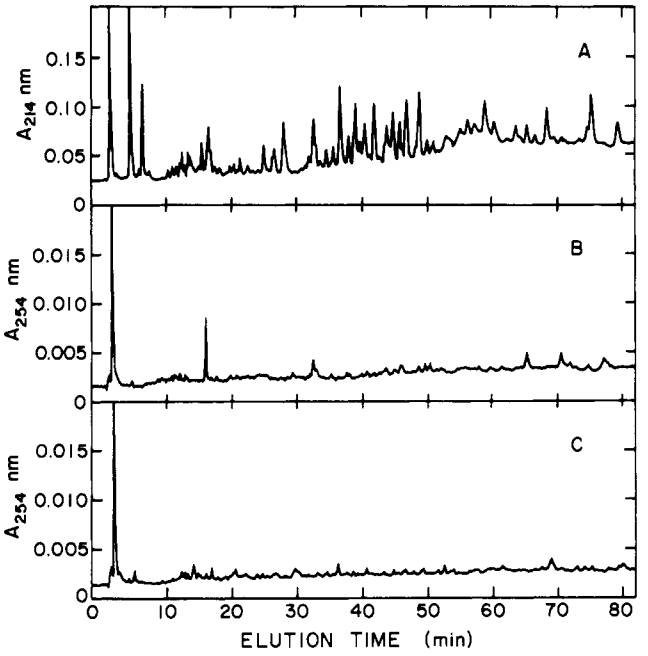

FIGURE 2: Peptide mapping of Lucifer yellow labeled CF_1. CF_1, either labeled with Lucifer yellow or untreated, was digested with trypsin, and the peptides were separated on HPLC. (A) CF_1 elution profile of Lucifer yellow modified CF_1 using 214-nm absorbance for detection. (B) Elution profile of the peptides of Lucifer yellow modified CF_1 using 254-nm absorbance for detection. (C) Control CF_1 elution profile using 254-nm absorbance for detection.

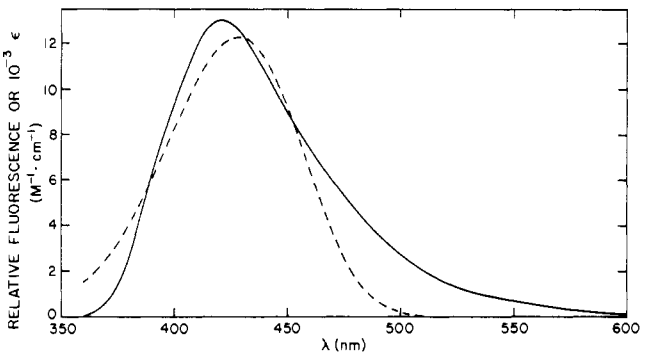

FIGURE 3: Spectral overlap of the fluorescence emission for DMSM covalently bound to CF_1 at either the dark-accessible site or the light site of CF_1 (solid line) and the absorbance spectrum of Lucifer yellow bound to CF_1 (dashed line).

from 0.75 to 1.0 mol of Lucifer yellow per mole of CF_1.

To determine if more than one residue is modified in the α subunits, CF_1 reacted with Lucifer yellow was digested with trypsin, and the peptides were separated by HPLC on a linear gradient of 0.1% phosphoric acid/0.1% phosphoric acid in acetonitrile, pH 2.0. A single peak containing Lucifer yellow is observed in the peptide elution profile monitored either by absorbance at 254 nm (Figure 2) or by fluorescence (not shown). The retention time of the modified peptide indicates that it is likely to be less than eight amino acids in length and suggests that the probe is selective for a unique amino acid residue on the α subunits.

Although Lucifer yellow can react with either sulfhydryl- or amine-containing residues, several observations indicate that the reactive site on the α subunit of CF_1 is the ϵ-amino group of a lysyl residue. The modification has a pH optimum above pH 9.0 and is decreased by pretreatment of CF_1 with either methyl acetimidate, a general amine reagent, or pyridoxal 5′-phosphate, a lysyl-specific reagent (not shown). In contrast, *N*-ethylmaleimide had no effect on the reactivity of CF_1 with Lucifer yellow, consistent with a previous report that the single sulfhydryl group in the α subunit (Deno et al., 1983) is inaccessible to modifying reagents (Nalin & McCarty, 1984). The amino group on the α subunit of CF_1 in solution that is especially reactive with Lucifer yellow does not react rapidly with the probe when CF_1 is membrane bound. Moreover, Lucifer yellow fails to react with the α subunits of CF_1 bound to CF_0, the membrane component of the H^+-ATPase (not shown).

Incorporation of 1 mol of Lucifer yellow/mol of CF_1 into the α subunit results in less than 10% inhibition of the ATPase activity of the enzyme.

Fluorescence Energy Transfer Measurements. The spectral properties of Lucifer yellow make it a useful probe for fluorescence energy transfer measurements. The absorbance spectrum of Lucifer yellow overlaps the fluorescence emission spectrum of DMSM, resulting in a donor–acceptor pair having an R_0 = 26–32 Å (Figure 3).

The efficiencies of fluorescence energy transfer between the sulfhydryl residues and bound Lucifer yellow were measured by comparing the fluorescence of enzyme specifically modified with Lucifer yellow on the α-subunit and DMSM either at the accessible sulfhydryl (dark site) or at the light-accessible site on the γ subunit with similarly labeled CF_1 containing no Lucifer yellow. The observed transfer efficiency normalized to the stoichiometry of Lucifer yellow labeling yields distances of 30 and 38 Å between bound Lucifer yellow and the dark and light site sulfhydryl residues, respectively (Table II).

The distance between bound Lucifer yellow and the thiols formed by reduction of the disulfide in the γ subunit was also determined by fluorescence energy transfer using DMSM as the donor. Since sulfhydryl residues exposed by reduction can also react with Lucifer yellow, care was taken to react the enzyme with *N*-ethylmaleimide following DMSM modification to prevent any subsequent Lucifer yellow incorporation into the γ subunit. SDS–polyacrylamide gel electrophoresis of the modified enzyme showed no Lucifer yellow fluorescence in the γ subunit. Within the resolution of the experiment ($E < 0.05$), no energy transfer could be detected between the Lucifer yellow reactive site and DMSM bound to the disulfide sulfhydryls. The absence of energy transfer between the disulfide

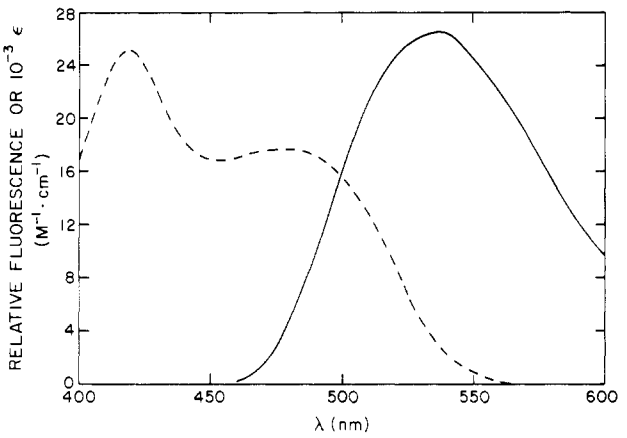

FIGURE 4: Absorption spectrum of TNP-ATP and fluorescence emission spectrum for Lucifer yellow. The dashed line shows the absorbance spectrum of TNP-ATP attached to site 1 and the solid line the fluorescence of Lucifer yellow attached to the α subunits of CF_1.

sulfhydryls and bound Lucifer yellow indicates that the bound probes are greater than 43 Å apart.

Distances between each of the three nucleotide binding sites on CF_1 and bound Lucifer yellow were obtained by using Lucifer yellow as the donor and TNP-ATP as the acceptor. The high quantum yield (0.36) and long-wavelength fluorescence (537 nm) of bound Lucifer yellow helped to offset the small spectral overlap (Figure 4), resulting in R_0 = 34.8 Å. Neither the quantum yield nor the steady-state polarization of the bound Lucifer yellow was affected by nucleotide binding.

After labeling with Lucifer yellow, specific binding of TNP-ATP to site 1 was accomplished by incubating labeled CF_1 with TNP-ATP for several hours in buffer lacking metal ions. Following passage of the enzyme through two successive centrifuge columns to remove free and dissociable nucleotides, only site 1 retained bound TNP-ATP. The stoichiometry of binding was 1.0 mol of TNP-ATP/mol of CF_1. The efficiency of energy transfer between Lucifer yellow and site 1 was then measured by comparing the fluorescence of CF_1 containing TNP-ATP to that containing no TNP-ATP. No energy transfer was observed within the resolution of the experiment. From the calculated R_0, this implies that the two sites are at least 57 Å apart.

Energy transfer between bound Lucifer yellow and TNP-ATP at site 2 was similarly measured after incubating Lucifer yellow labeled CF_1 with TNP-ATP in buffer containing Mg^{2+}. Under these conditions, both site 1 and site 2 bind TNP-ATP, and nucleotide binding stoichiometries of 1.9–2.0 mol of TNP-ATP/mol of CF_1 were obtained. The efficiency of energy transfer to site 2 was 0.43, resulting in a calculated distance of 36 Å from bound Lucifer yellow to site 2.

Direct measurements of the energy transfer between Lucifer yellow and TNP-ATP at site 3 could be obtained by titrating site 3 with TNP-ATP and monitoring the decrease in Lucifer yellow fluorescence after first saturating site 2 with either TNP-ATP or ATP. The results of the titrations when site 2 is blocked with TNP-ATP are shown in Figure 5. Similar results were obtained for site 2 containing ATP. At each concentration of TNP-ATP, the observed fluorescence was corrected for inner filter effects and for dilution of the sample. The concentrations of TNP-ATP used in the titrations were 0.2–17 μM. Since the increased quenching observed in the titrations represented energy transfer from binding to a single nucleotide site, the data could be fit to the binding equation

$$Q_{DA}/Q_D = 1 - E[EL]/[E_0] \quad (3)$$

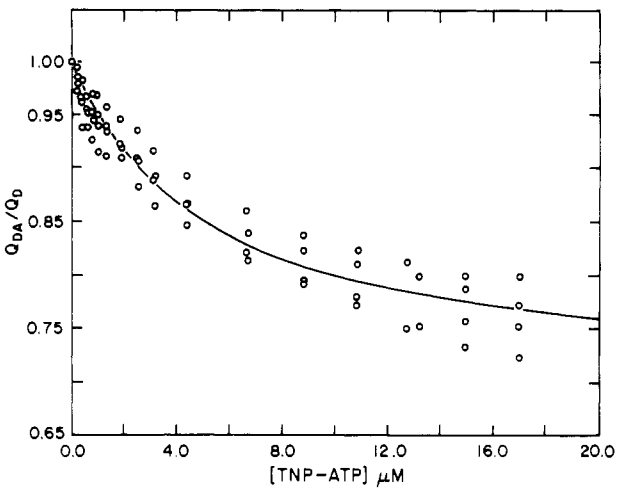

FIGURE 5: Titration of site 3 on CF_1 labeled with Lucifer yellow using TNP-ATP. Fluorescence quenching of Lucifer yellow bound to CF_1, Q_{DA}/Q_D, vs. the total concentration of TNP-ATP. The points represent four separate titrations. All titrations were performed with 0.3 mg/mL CF_1 in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, and 2 mM EDTA at 25 °C. The curve is a nonlinear least-squares fit of the data to eq 3 as described in the text.

where E is the relative energy transfer efficiency when the site is fully saturated, and [EL] is the concentration of bound ligand. [EL] can be readily calculated from the dissociation constant for the enzyme–nucleotide complex and the total enzyme concentration, $[E_0]$ (Snyder & Hammes, 1984). A nonlinear least-squares fit of the data reveals a K_d = 4.6 ± 0.6 μM, in agreement with the binding affinity of site 3 for nucleotide reported previously (Cantley & Hammes, 1975; Bruist & Hammes, 1981; Cerione & Hammes, 1982), and an E = 0.30 ± 0.02. The total transfer efficiency with both sites 2 and site 3 fully occupied is thus 0.60. According to eq 1 and the distance calculated above to nucleotide site 2, this efficiency corresponds to a distance of 37 Å between Lucifer yellow and site 3.

The distance between the Lucifer yellow reactive site on the α subunit and the sulfhydryl residue of the ε subunit was determined with DMSM as the donor. The ε subunit was isolated from CF_1 by M. L. Richter (Richter et al., 1984) and then modified. Modification of isolated ε subunit, followed by reconstitution with $CF_1(-\epsilon)$, permits selective labeling without introducing label into the γ subunit. After labeling with DMSM, Lucifer yellow was incorporated into the α subunit, and the fluorescence of DMSM in the absence and presence of bound Lucifer yellow was measured. The observed transfer efficiency between DMSM and Lucifer yellow, E = 0.16/mol of Lucifer yellow, yields a distance between the ε-subunit sulfhydryl and the Lucifer yellow site of 42 Å.

Discussion

In this paper, we have shown that Lucifer yellow reacts with a unique site, probably an amino group, in soluble CF_1. Although CF_1 probably contains three copies of the α subunit, our fluorescence energy transfer experiments indicate that Lucifer yellow reacts with a specific α subunit, rather than with a group on each α subunit, to the extent of about 0.3 mol per mole of α subunit. The distances between Lucifer yellow and probes bound to seven discrete sites on CF_1 were estimated. These distances converge to a single location (Figure 6), arguing strongly for a unique site on a single α subunit that has an intrinsic high reactivity with Lucifer yellow. The kinetics and extent of labeling with Lucifer eyllow are also most consistent with a unique site on an α subunit. The 10-nm blue shift in the fluorescence emission maximum of Lucifer yellow

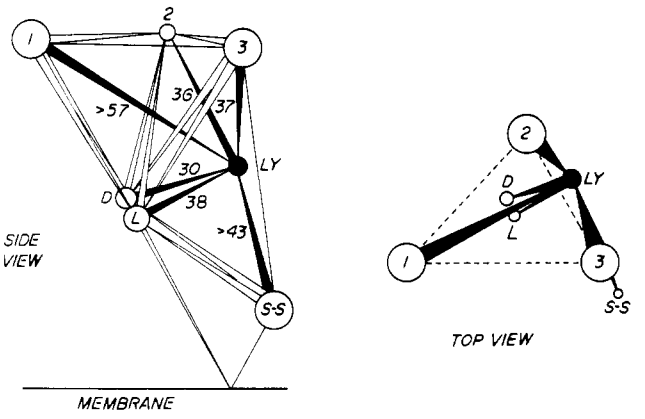

FIGURE 6: Model for CF_1 showing the distances between the Lucifer yellow site (LY) and other sites on CF_1. The positions of all other sites are based on the work of Snyder & Hammes (1984, 1985). 1, 2, and 3 stand for nucleotide binding sites 1, 2, and 3. L, D, and S-S stand for the cysteinyl residues of the γ subunit.

bound to CF_1 relative to that of the probe in aqueous solution indicates that the probe is in a hydrophobic environment. The high degree of polarization of bound Lucifer yellow fluorescence suggests that the probe has limited rotational freedom.

The distances between bound Lucifer yellow on an α subunit and various sites on CF_1 labeled independently were incorporated into a model (Figure 6). The agreement between the spatial relationships determined in this study and previous determinations (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985) is within the experimental limitations of the method. In addition, the measured distance between the bound Lucifer yellow and the sulfhydryl site of the ϵ subunit is consistent with other distance determinations involving the ϵ-sulfhydryl (B. Snyder and M. L. Richter, unpublished results). The site on the α subunit that reacts with Lucifer yellow is approximately equidistant from nucleotide binding sites 2 and 3 but is very far from nucleotide site 1. The three nucleotide sites appear to form a triangle that is approximately parallel to the plane of the membrane (Snyder & Hammes, 1984). The Lucifer yellow site would then be closer to the membrane than the nucleotide sites. This result does not imply that the α subunits are positioned below the β subunits since both subunits are sufficiently large to span the distances involved.

Our finding that CF_1 contains a structurally unique Lucifer yellow binding site on one of three α subunits provides strong evidence that the soluble enzyme is structurally asymmetric. Since there is a single copy of the γ subunit, and probably of the δ and ϵ subunits as well, there are likely to be specific sites at which these smaller subunits interact with the α and β subunits. Lucifer yellow may react with a site on an α subunit made accessible by the interactions of that α subunit with one or more of the smaller subunits. In this regard, it may be relevant that the shortest distance measured is between bound Lucifer yellow and fluorescent maleimide bound to the accessible sulfhydryl group of the γ subunit. The α subunit of CF_1 in the thylakoids or bound to F_0 is resistant to attack by Lucifer yellow. Although the α subunits of CF_1 in solution are rapidly cleaved by trypsin and other proteases (Moroney & McCarty, 1982), α is cleaved very slowly in membrane-bound CF_1. In addition, CF_1 containing clipped α subunits will not bind to thylakoids from which the CF_1 has been removed (Patrie & McCarty, 1984). Thus, the α subunits may be involved in binding and could interact with a subunit(s) of F_0. These interactions may explain the decrease in sensitivity of the α subunit to attack by proteases or Lucifer yellow that occurs when CF_1 is bound to F_0.

Asymmetry induced by organization of the subunits and ligand-induced asymmetry by substrates and products may play a role in maintaining CF_1 in its active conformations and its catalysis (Kohlbrenner & Boyer, 1983). Although the reaction of CF_1 with Lucifer yellow was influenced by nucleotides, turnover of the enzyme did not appear to enhance the reactivity of the α subunits to the probe (C. M. Nalin, unpublished results). Thus, at least with respect to the site on α, catalysis does not seem to affect the asymmetry of the enzyme.

Future experiments with the Lucifer yellow modified enzyme will focus on the role of the unique α subunit in energy coupling and on changes in this subunit that occur when CF_1 binds to F_0.

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Structural Organization of Chloroplast Coupling Factor†

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ABSTRACT: Fluorescence resonance energy transfer measurements have been used to construct spatial maps for the accessible sulfhydryl of the γ subunit (dark site) and the essential tyrosine residue of the β subunits relative to previously mapped sites on the H^+-ATPase from chloroplasts. The extent of energy transfer was measured between a coumarinylmaleimide derivative reacted covalently at the dark site and acceptor species selectively bound at the γ-disulfide and the three nucleotide binding sites of the solubilized coupling factor complex. The nucleotide energy acceptor was 2′(3′)-(trinitrophenyl)adenosine triphosphate, and the γ-disulfide site was labeled with fluoresceinylmaleimide. The dark-site sulfhydryl also was labeled with pyrenylmaleimide which served as an energy donor for 7-chloro-4-nitro-2,1,3-benzoxadiazole reacted at the β-tyrosine sites. Similar measurements were also made with pyrenylmaleimide covalently attached to the γ-sulfhydryl accessible only under energized conditions on the thylakoid membrane surface (light site). The observed transfer efficiencies indicate that the dark-site sulfhydryl is ~45 Å from all three nucleotide sites and 41-46 Å from the γ-disulfide site. The average distances separating the essential β-tyrosines and the light- and dark-site sulfhydryls are 38 and 42 Å, respectively. (In calculating these distances, random orientation of the donor-acceptor dipoles was assumed.) The results are consistent with a previously described structural model of the intact enzyme and can be used to gain insight into the overall structural organization of α-, β-, and γ-polypeptides within the coupling factor.

The dicyclohexylcarbodiimide-sensitive H^+-ATPase from spinach chloroplasts catalyzes the synthesis of ATP through a coupled transport of protons across the thylakoid membrane. The extrinsic portion of this coupling factor complex, chloroplast coupling factor 1 (CF_1),[1] can be readily stripped from the thylakoid membrane through treatment with EDTA (Lien & Racker, 1971). In its solubilized form, CF_1 catalyzes the hydrolysis of ATP (Farron & Racker, 1970). In order for this hydrolysis to be expressed, the enzyme, which is latent when isolated, must first be activated, typically through treatment with heat and/or thiol reducing agents (Farron & Racker, 1970). CF_1 contains five different types of polypeptides (α, β, γ, δ, and ϵ, in decreasing order of size; Pick & Racker, 1979) and has a molecular weight of 400000 (Moroney et al., 1983). The polypeptide chain stoichiometry of the three largest subunits is probably $\alpha_3\beta_3\gamma$ (Moroney et al., 1983).

Three distinct nucleotide binding sites have been characterized on CF_1 (Bruist & Hammes, 1981). The site designated as site 1 contains tightly bound ADP which cannot be removed by extensive dialysis but exchanges readily with medium nucleotides; site 2 binds ATP tightly in the presence of Mg^{2+}, and site 3 binds nucleotides reversibly under all conditions with dissociation constants in the micromolar range. Studies using nucleotide analogues as photoaffinity reagents suggest that all three nucleotide binding sites are in similar locations at interfaces of the α- and β-polypeptide chains (Bruist & Hammes, 1981; Kambouris & Hammes, 1985). Four cysteinyl residues on the γ subunit of CF_1 have been characterized on the basis of their reactivity with various alkylating reagents (Nalin et al., 1983; Nalin & McCarty, 1984; Moroney et al., 1984). One cysteine is accessible under all conditions (dark-site sulfhydryl), while another can be modified only under energized conditions on the thylakoid membrane (light-site sulfhydryl). The two remaining cysteinyl groups form a disulfide bond which can be reduced by incubating the enzyme with excess dithiothreitol (disulfide site). Reduction

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; TNP-ATP, 2′(3′)-(trinitrophenyl)-adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide.

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