In addition to potential sensitivity, many of the dyes have properties that may make them useful as reporters of other membrane properties or processes. They are strongly fluorescent when bound and practically nonfluorescent in water. Their large Stokes shifts make it possible to use broad bandwidths for excitation and emission. They have lipid-like structures, which should not cause severe perturbations to the membrane structure; this is especially true for the zwitterionic probes. They are chemically and photochemically quite stable and are insensitive to pH. Finally, they bind with their transition moments perpendicular to the membrane surface, making them attractive complements to the class of long-chain cyanine dyes that have been widely used in membrane studies.

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Binding Stoichiometry and Structural Mapping of the ϵ Polypeptide of Chloroplast Coupling Factor 1†

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ABSTRACT: Fluorescent probes were attached to the single sulfhydryl residue on the isolated ϵ polypeptide of chloroplast coupling factor 1 (CF_1), and the modified polypeptide was reconstituted with the ϵ-deficient enzyme. A binding stoichiometry of one ϵ polypeptide per CF_1 was obtained. This stoichiometry corresponded to a maximum inhibition of the Ca^{2+}-dependent ATPase activity of the enzyme induced by ϵ removal. Resonance energy transfer between the modified ϵ polypeptide and fluorescent probes attached to various other sites on the enzyme allowed distance measurements between these sites and the ϵ polypeptide. The ϵ-sulfhydryl is nearly equidistant from both the disulfide (23 Å) and the dark-accessible sulfhydryl (26 Å) of the γ subunit. Measurement of the distance between ϵ and the light-accessible γ-sulfhydryl was not possible due to an apparent exclusion of modified ϵ from ϵ-deficient enzyme after modification of the light-accessible site. The distances measured between ϵ and the nucleotide binding sites on the enzyme were 62, 66, and 49 Å for sites 1, 2, and 3, respectively. These measurements place the ϵ subunit in close physical proximity to the sulfhydryl-containing domains of the γ subunit and approximately 40 Å from the membrane surface. Enzyme activity measurements also indicated a close association between the ϵ and γ subunits: ϵ removal caused a marked increase in accessibility of the γ-disulfide bond to thiol reagents and exposed a trypsin-sensitive site on the γ subunit. Either disulfide bond reduction or trypsin cleavage of γ significantly enhanced the Ca^{2+}-ATPase activity of the ϵ-deficient enzyme. Thus, the ϵ and γ polypeptides of coupling factor 1 are closely linked, both physically and functionally.

Chloroplast coupling factor 1 (CF_1)[1] catalyzes ATP synthesis by utilizing energy derived from a transmembrane proton gradient. The enzyme is also a latent ATPase which may be activated by a number of different treatments both on and off the membrane [cf. McCarty & Moroney (1985)]. CF_1 is composed of five different polypeptides designated α–ϵ in order

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; $CF_1(-\epsilon)$, CF_1 lacking the ϵ polypeptide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine 5′-triphosphate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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of decreasing molecular weight. The probable polypeptide stoichiometry is $\alpha_3\beta_3\gamma\delta\epsilon$ although the exact number of copies of the smaller polypeptides (δ and ϵ) has been questioned because of possible losses during CF_1 preparation (Binder et al., 1978; McCarty & Moroney, 1985).

Nucleotide binding and catalysis appear to be restricted to the α and β polypeptides of CF_1 (Kambouris & Hammes, 1985). The γ polypeptide, although required for ATP synthesis, probably functions as a regulatory subunit and may be involved in proton translocation (Weiss & McCarty, 1977; Moroney et al., 1983). The δ polypeptide was originially thought to be required for binding CF_1 to the hydrophobic portion of the enzyme complex (CF_0) by analogy to the δ polypeptide of bacterial F_1 (Abrams et al., 1976; Mollinedo et al., 1980; Yoshida et al., 1977). Recent studies (Patrie & McCarty, 1984), however, suggest that δ is not absolutely required either for CF_1 binding to the membrane or for ATP synthesis, although its presence may be necessary for full functional integrity of the coupling factor complex. Nelson et al. (1972) were first to propose that the smallest polypeptide (ϵ) may act as an ATPase inhibitor of CF_1. This was confirmed recently by studies utilizing a method for the selective removal and isolation of ϵ from CF_1 (Richter et al., 1984). ϵ removal resulted in a permanent activation of the Ca^{2+}-dependent ATPase activity of the enzyme together with a complete loss of the ability of CF_1 to block the free flow of protons across the membrane and to synthesize ATP. All of these effects were fully reversed by recombining ϵ with ϵ-deficient CF_1. Although the ϵ of CF_1 is similar to that of *Escherichia coli* F_1 [cf. Dunn & Heppel (1981)] in that they are both ATPase inhibitors, the chloroplast ϵ is not required for binding CF_1 to the membrane (Richter et al., 1984; Patrie & McCarty, 1984), in contrast to *E. coli* F_1.

Selective removal and reconstitution of the ϵ polypeptide have allowed the specific chemical modification of the single ϵ-sulfhydryl residue with fluorescent probes. Studies are described in this paper in which CF_1 reconstituted with modified ϵ was used for determining the binding stoichiometry of ϵ and for structural mapping of the ϵ subunit within CF_1. The results indicate a binding stoichiometry of one ϵ per CF_1. Structural mapping, which was achieved by measuring resonance energy transfer between the modified ϵ subunit and fluorescent probes attached to other sites on CF_1, indicates that ϵ is well removed from the catalytic site(s) on the α/β subunits but is in close physical proximity to the sulfhydryl-containing domains of the γ polypeptide. This latter finding is supported by the observation that removal of ϵ from CF_1 greatly facilitated the accessibility of the γ subunit to external reagents.

Materials and Methods

Chemicals. ATP (vanadium free) was from Sigma Chemical Co. TNP-ATP and all fluorescent maleimide derivatives were from Molecular Probes, Inc. Quinine sulfate was obtained from Aldrich Chemical Co. and TPCK–trypsin from Worthington. All other chemicals were high-quality commercial grades, and all aqueous solutions were prepared with deionized water.

Preparation of CF_1, ϵ, and ϵ-Deficient CF_1. CF_1 was prepared from fresh market spinach (Lien & Racker, 1971; Binder et al., 1978). An additional wash of the thylakoids with 10 mM NaCl was included to remove contaminating ribulosebisphosphate carboxylase (Moroney & McCarty, 1982). The purified enzyme 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 or, for smaller volumes, through column centrifugation (Penefsky, 1977). Molar concentrations of CF_1 were determined by using a modification of the Lowry method (Peterson, 1977) or an extinction coefficient of 0.483 cm^2/mg at 277 nm (Bruist & Hammes, 1981) and a molecular weight of 400 000 (Moroney et al., 1983).

Purified ϵ polypeptide and ϵ-deficient CF_1 [$CF_1(-\epsilon)$] were prepared essentially as described previously (Richter et al., 1984). The alcohol mixture used for ϵ isolation (ϵ isolation buffer) contained glycerol (30% v/v), ethanol (20% v/v), 25 mM Tris-HCl (pH 8.0), 3 mM ATP, and 20 mM NaCl. The presence of NaCl in the alcohol mixture caused the ϵ polypeptide to elute from the DEAE-cellulose column in a more concentrated form.

Ca^{2+}-dependent ATP hydrolysis was measured for 2 min at 37 °C in the presence of 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $CaCl_2$, and 5 μg/mL enzyme preparation. P_i was determined spectrophotometrically (Taussky & Shorr, 1953).

Chemical Modification and Reconstitution of the ϵ Polypeptide. The ϵ-sulfhydryl was modified by incubating the isolated ϵ (100–150 μg/mL in the alcohol mixture) with 50 μM fluorescent maleimide for 60 min at room temperature. Unreacted probe was removed by adding 1–2 mM *N*-acetylcysteine and incubating the mixture for at least 30 min. $CF_1(-\epsilon)$ [1–3 mg in 25 mM Tris-HCl (pH 8.0), 1 mM ATP, and 0.4 M NaCl] was mixed with an excess of modified ϵ and 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM ATP such that the final ethanol concentration was less than 7% (v/v). After incubation at room temperature for 5 min, the mixture was passed through a Sephadex G-50 column (2 × 22 cm) to remove free probe and unbound ϵ. The protein was concentrated by binding it to a small column (0.7 × 2 cm) of DEAE-cellulose and eluting it with 25 mM Tris-HCl (pH 8.0), 1 mM ATP, and 0.4 M NaCl. Glycerol was added to 20% (v/v) for storage for short times at room temperature or longer times at 0–4 °C. The enzyme was passed through a Sephadex G-50 centrifuge column immediately before spectroscopic analysis. Specificity of the labeling was routinely tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Chua, 1980). Fluorescent probes were dissolved in ethanol or dimethyl sulfoxide shortly before use. Aged solutions of CPM heavily labeled all subunits of CF_1 even after the probe had been reacted with *N*-acetylcysteine.

Chemical Modification of CF_1. CF_1 was selectively modified at the "dark"- or "light"-accessible γ-subunit sulfhydryl residues as described elsewhere (Snyder & Hammes, 1984, 1985). The ϵ polypeptide was removed from these preparations and replaced with modified ϵ. The γ-disulfide sulfhydryls were modified on CF_1 already reconstituted with modified ϵ subunit. The enzyme [2.5 μM in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA] was first incubated at room temperature with 2 mM *N*-ethylmaleimide for 15 min to block reactive sulfhydryls. Dithiothreitol was added to 50 mM and the mixture incubated at room temperature for 3–5 h to reduce the γ-disulfide (Nalin & McCarty, 1984). Excess dithiothreitol was removed by column centrifugation (Sephadex G-50) and the enzyme treated for 5 min with 50 μM fluorescent maleimide. Unreacted probe was removed by one to two consecutive column centrifugations.

Procedures for specific labeling of each of the nucleotide binding sites of CF_1 with TNP-ATP have been described elsewhere (Cerione & Hammes, 1982; Snyder & Hammes, 1984). The site designated as site 1 contains tightly bound nucleotide which exchanges readily with medium nucleotide; site 2 binds ATP derivatives tightly in the presence of Mg^{2+}; site 3 binds nucleotides reversibly under all conditions with

dissociation constants in the micromolar range (Bruist & Hammes, 1981).

Probe concentrations and labeling stoichiometries for each probe were calculated by use of extinction coefficients of 3.75 × 10^4 M^{-1} cm^{-1} at 343 nm for pyrenylmaleimide (Holowka & Hammes, 1977), 3.02 × 10^4 M^{-1} cm^{-1} at 387 nm for CPM (Sippel, 1981), 7.08 × 10^4 M^{-1} cm^{-1} at 495 nm for fluoresceinylmaleimide (Cerione et al., 1983), 2.64 × 10^4 M^{-1} cm^{-1} at 408 nm for free TNP-ATP (Hiratsuka & Uchida, 1973), and 2.51 × 10^4 M^{-1} cm^{-1} at 418 nm for bound TNP-ATP (Cerione & Hammes, 1982). In the calculation of stoichiometries, corrections were made for probe absorbance at 277 nm, CF_1 light scattering at all wavelengths, and probe–probe spectral overlaps.

ε Binding Stoichiometry Measurements. The method of continuous variation (Job, 1928; Asmus, 1961) was used to determine the amount of ε required for full inhibition of the Ca^{2+}-dependent ATPase activity of the ε-deficient enzyme. The total concentration of $CF_1(-\epsilon)$ plus ε subunit was held constant while the relative concentration (mole fraction) of ε was varied between 0 and 1. Reconstitution mixtures (170 μL) contained 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM ATP and 0–150 μg of $CF_1(-\epsilon)$; 0–70 μL of stock ε solution (80.7 μg/mL) or 0–70 μL of the ε isolation buffer was added, giving a final volume of 240 μL for each sample. Control mixtures for each mole fraction were identical except that ε was omitted. After at least 5-min incubation at room temperature, aliquots containing 5 μg of $CF_1(-\epsilon)$ were taken for Ca^{2+}-ATPase assay. The difference between the total ATPase activity and that of the control gave a measure of the extent of inhibtion by ε at each mole fraction.

CF_1 was also titrated with modified ε, and binding and ATPase inhibition were measured. The ε-sulfhydryl was modified with pyrenylmaleimide as described above. Free probe was removed by passage through a column (0.7 cm × 8 cm) of Sephadex LH-20 at 4 °C. The ε isolation buffer was used to equilibrate the column and to elute the modified ε polypeptide. Reconstitution mixtures contained, in 100-μL volumes, 80 μg of $CF_1(-\epsilon)$ in 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM ATP, and 40 μL of the alcohol mixture substituted with 0–40 μL of the modified ε solution. After incubation for 5 min at room temperature, unbound ε was removed by column centrifugation as follows: Sephadex G-75 centrifuge columns (3 mL, Fisher Scientific) were equilibrated with 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM ATP and centrifuged (60 s, 190*g* average). Each 100-μL sample was loaded onto a column followed by 300 μL of the equilibration buffer, and centrifugation was repeated. Column eluates were assayed for pyrene fluorescence, protein (Lowry et al., 1951), and Ca^{2+}-ATPase activity. Protein recovery after centrifugation was approximately 40–50%. Duplicate samples containing normal CF_1 instead of $CF_1(-\epsilon)$ were processed to correct for nonspecific adsorption of modified ε by CF_1. Typically, such corrections amounted to less than 15% of the total fluorescence of the reconstituted CF_1 at saturating ε concentrations. The amount of ε polypeptide bound was determined by comparison of the fluorecence of column eluates to that of a standard curve prepared by adding increasing amounts of modified ε to an excess of $CF_1(-\epsilon)$ in 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM ATP. The standard curve was linear over the concentration range used.

To determine the protein concentration of the ε stock solution, an aliquot was diluted 10-fold with distilled H_2O, and trichloroacetic acid was added to a final concentration of 10% (w/v). The solution was centrifuged at 10000*g* for 30 min, and the precipitated ε was resuspended in a small volume of 50% (v/v) ethanol. The protein concentration of this solution was determined by amino acid analysis using a Millipore/Waters PICO-TAG amino acid analysis system. The amino acid composition of ε deduced from this analysis agreed well with that derived from the published amino acid sequence (Zurawski et al., 1982). The methods of Lowry et al. (1951) and Bradford (1976) gave protein concentration estimates 17% higher and 43% lower, respectively, than those determined by amino acid analysis. Lower values using the Bradford method were expected because of the relatively low adsorption of Coomassie dye by ε (Binder et al., 1978). This method was, however, used to determine the recovery of ε (78%) after trichloroacetic acid precipitation since the other two methods were sensitive to the glycerol present in the stock ε solution. The concentration of ε determined by amino acid analysis, after adjustment for recovery, was used as a standard in all experiments, together with a molecular weight for ε of 14700 (Zurawski et al., 1982).

Spectroscopic Measurements. Absorbance measurements were made with either a Beckman DU-7 or a Cary 118 spectrophotometer. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. The measured steady-state polarizations were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating. Quantum yields for the fluorescent-labeled CF_1 samples were calculated by a comparative method (Parker & Reese, 1966). Quinine sulfate in 0.1 N H_2SO_4 was used as a fluorescence standard and was assumed to have a quantum yield of 0.70 (Scott et al., 1970). Binding stoichiometry fluorescence measurements were made with a Farrand fluorescence spectrophotometer.

Time-Resolved Fluorescence Measurements. Fluorescence lifetimes were measured with an ORTEC 9200 nanosecond fluorescence spectrophotometer as described previously (Matsumoto & Hammes, 1975). Pyrenylmaleimide-labeled CF_1 samples were excited with light from a spark gap flash lamp that was vertically polarized and passed through a Ditric 340-nm three-cavity band-pass filter. The fluorescence emission was either vertically or horizontally polarized and passed through a Corning CSO-51 cut-off filter with 50% transmittance at 385 nm. Background and scattering corrections were determined with an equivalent concentration of unlabeled CF_1. The time-resolved fluorescence, $F(t)$, was calculated as

$$F(t) = V(t) + 2H(t) \quad (1)$$

where $V(t)$ and $H(t)$ are the spectra collected with the emission polarizer oriented vertically and horizontally, respectively. The data were fit through a weighted nonlinear least-squares analysis in which the lamp pulse is convoluted by a numerical procedure (Munro et al., 1979; Anderson & Hammes, 1983). The lamp pulse was determined from the light scattering of a 0.1% Ludox solution.

Fluorescence Resonance Energy Transfer Measurements. Fluorescence resonance energy transfer measurements were performed as described previously (Snyder & Hammes, 1984, 1985). For all cases, the donor fluorescence was corrected for the measured probe absorbance, protein light scattering, and acceptor stoichiometries. Efficiencies of energy transfer were obtained from the relationship

$$E = 1 - Q_{DA}/Q_D = 1 - \tau_{DA}/\tau_D \quad (2)$$

where Q_{DA}/Q_D and τ_{DA}/τ_D are the ratios of the donor quan-

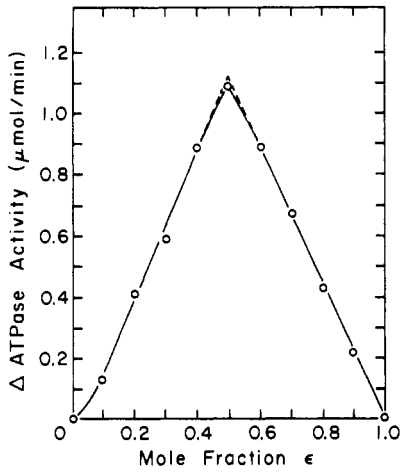

FIGURE 1: Job plot for the binding of the ϵ polypeptide to $CF_1(-\epsilon)$. $CF_1(-\epsilon)$ and ϵ were mixed in different proportions while the total protein concentration (1.7×10^{-6} M) was kept constant. ΔATPase activity is the difference between the total Ca^{2+}-ATPase activity of each sample and that of a control without ϵ added.

tum yields and fluorescence lifetimes, respectively, in the presence (DA) and absence (D) of acceptor compounds. All reported transfer efficiencies are the average of at least two measurements, with standard deviations of <4% from the average value. Distances between donor and acceptor sites were calculated from the relationship

$$E = \sum_{i=1}^{N_A}(R_0/R_i)^6/[1 + \sum_{i=1}^{N_A}(R_0/R_i)^6] \quad (3)$$

where N_A is the number of acceptors contributing to the quenching of a single donor, R_i is the distance between the donor and the ith acceptor, and R_0 is the distance at which the transfer efficiency is 0.5 for a single donor–acceptor pair. Equation 3 assumes that each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of CF_1. For multiple acceptors, all but one of the donor–acceptor distances had previously been measured such that eq 3 contained only one unknown. For the case of the reduced γ-disulfide, the distances to each individual sulfhydryl were assumed to be equal (Snyder & Hammes, 1984). In eq 3, R_0 is given by (Förster, 1959)

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

where n is the refractive index of the medium, J is the spectral overlap integral, and κ^2 is an orientation factor for dipolar coupling between donors and acceptors. The value of κ^2 was assumed to be $^2/_3$, the dynamic average. The maximum uncertainties in the calculated distance due to this assumption are likely to be no worse than ±10% (Snyder & Hammes, 1985). The contributions of inner filter effects and trivial transfer to the steady-state quenching ratios were calculated directly from the measured acceptor absorbance at the donor excitation and emission wavelengths, respectively. These corrections were typically less than 5% of the total quenching observed.

RESULTS

ϵ Binding Stoichiometry Measurements. An estimation of the functional binding stoichiometry of the ϵ polypeptide was made by using the method of continuous variation (Job, 1928). This method determined the mole fraction of ϵ required to give maximum inhibition of the ATPase activity of the ϵ-deficient enzyme. Plotting the difference between the total Ca^{2+}-ATPase activities in the absence or presence of ϵ at different mole fractions resulted in the symmetrical curve shown in Figure 1. The intersection of the linear portions of this curve occurs at a mole fraction of 0.5, indicating a binding stoichiometry of one ϵ polypeptide per CF_1 to give maximum inhibition. The sharpness of this titration indicates a very high affinity of $CF_1(-\epsilon)$ for ϵ. An estimate of the dissociation constant for this interaction from these data gives an upper limit of 8×10^{-10} M.

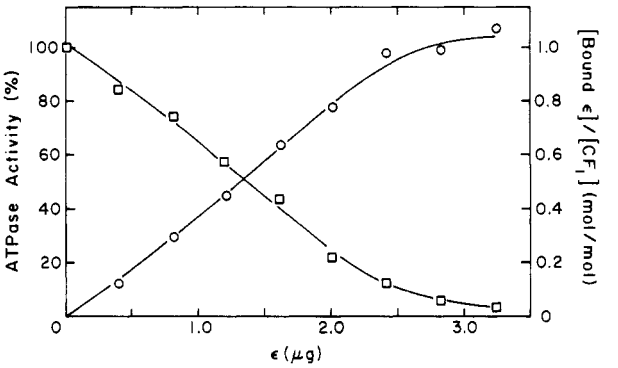

FIGURE 2: Binding of modified ϵ polypeptide to $CF_1(-\epsilon)$. Pyrenylmaleimide-labeled ϵ (0.44 mol of probe/mol of ϵ) was added, in the amounts shown, to $CF_1(-\epsilon)$. Bound ϵ and Ca^{2+}-ATPase activities were measured as described in the text, after removal of nonspecifically bound ϵ by column centrifugation. The maximum Ca^{2+}-ATPase activity (100%) was 16 μmol of P_i formed min^{-1} [mg of $CF_1(-\epsilon)$]$^{-1}$.

Titration of pyrenylmaleimide-labeled ϵ with the ϵ-deficient enzyme gave an essentially identical result (Figure 2). These data show a very strong correlation between ϵ binding and inhibition of the ATPase activity, 50% inhibition occurring at a stoichiometry of 0.5 mol of ϵ per mole of enzyme. Moreover, both curves tended to saturate at a stoichiometry close to one, suggesting a maximum of only one ϵ polypeptide per CF_1. These results do not reflect a preferential binding of the unmodified ϵ polypeptide since both modified and unmodified polypeptides inhibited Ca^{2+}-ATPase to the same extent (data not shown). Finally, the $CF_1(-\epsilon)$ reconstituted with saturating amounts of ϵ appeared to have a normal complement of ϵ as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see Figure 6, lane 5).

Energy Transfer Measurements. The existence of a single copy of the ϵ polypeptide and hence a single ϵ-sulfhydryl site per CF_1 molecule allows fluorescence resonance energy transfer techniques to be used to investigate, unambiguously, the spatial relationships between the ϵ-sulfhydryl site and previously mapped sites within the enzyme. The ϵ-sulfhydryl was specifically modified with appropriate fluorescent maleimides to serve as acceptors and/or donors for labels located at the two γ-sulfhydryls, the reduced γ-disulfide, and the three nucleotide binding sites of CF_1.

Initial attempts at resonance energy transfer measurements involving the ϵ-sulfhydryl site were made by using the coumarinylmaleimide derivative CPM. The fluorescence properties of the CPM-labeled ϵ-sulfhydryl on the reconstituted enzyme are summarized in Table I. Its high polarization, indicative of a restrictive environment, and high quantum yield are similar to those found for CPM located at the two γ-sulfhydryls and the reduced γ-disulfide sites of CF_1; however, its emission maximum (474 nm) is red shifted 4 nm relative to the CPM-modified γ-sulfhydryls (Snyder & Hammes, 1984, 1985). The fluorescence emission spectrum of CPM at the ϵ-sulfhydryl overlaps well with the absorption spectrum of TNP-ATP selectively bound to the nucleotide binding sites of CF_1 (see Figure 3). The value of R_0 for this donor–acceptor pair is quite large (44.8 Å), permitting the measurement of long distances. Labeling stoichiometries at the ϵ-sulfhydryl

Table I: Energy Transfer Parameters for CF_1[a]

donor	location	fluorescence max (nm)	Q_D	P^b	acceptor	location	absorbance max (nm)	P^b	R_0 (Å)[c]
CPM	ε	474	0.71	0.37	TNP-ATP	N1–N3	418/480		44.8
CPM	ε	474	0.71	0.37	FM	diSH	495	0.18	50.6
PM	ε	375/395	0.12	0.25	CPM	diSH	387	0.28	31.2
PM	light	375/395	0.30	0.25	CPM	ε	387	0.37	36.3
PM	dark	375/395	0.30	0.24	CPM	ε	387	0.37	36.3

[a] The words dark and light are the dark and light site γ-sulfhydryls, diSH is the γ-disulfide, ε is the sulfhydryl of the ε polypeptide, N1–N3 are nucleotide binding sites 1–3, PM is pyrenylmaleimide, and FM is fluoresceinylmaleimide. [b] Steady-state polarization measured at fluorescence excitation and emission maxima. [c] Calculated with eq 4 and the spectral properties of the donor and acceptor species. The value of κ^2 is assumed to be $^2/_3$.

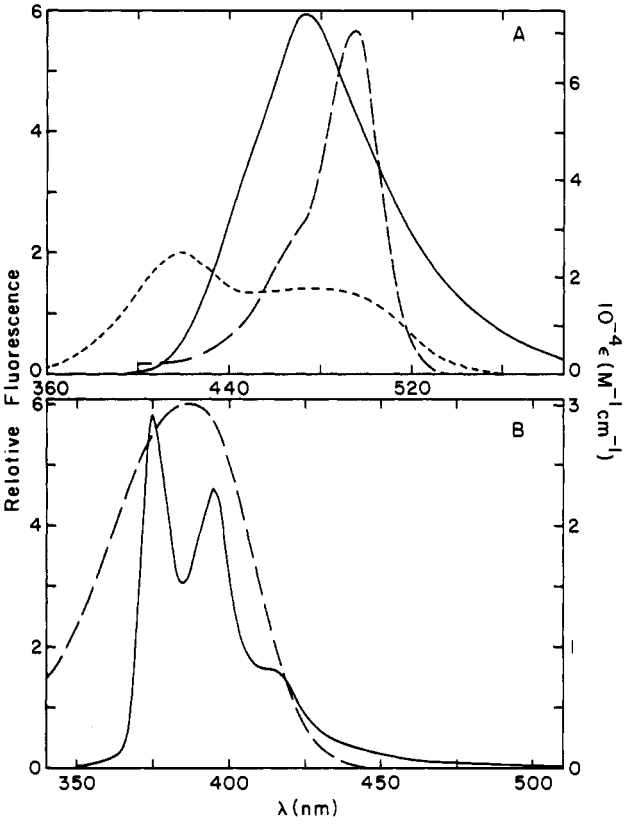

FIGURE 3: Spectral overlap of fluorescent donor–acceptor pairs. (A) Fluorescence emission for CPM (—) covalently bound to the ε-sulfhydryl on CF_1; extinction coefficients (ε) for fluoresceinylmaleimide (– –) covalently bound to the γ-disulfide sulfhydryls and TNP-ATP (– – –) bound to the nucleotide sites of CF_1. (B) Fluorescence emission for pyrenylmaleimide (—) covalently bound to the ε-sulfhydryl on CF_1; extinction coefficient for CPM (– –) covalently bound to the reduced disulfide of γ. The fluorescence excitation was 387 nm for CF_1–CPM and 343 nm for CF_1–pyrenylmaleimide. All spectra were recorded in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA at 25 °C.

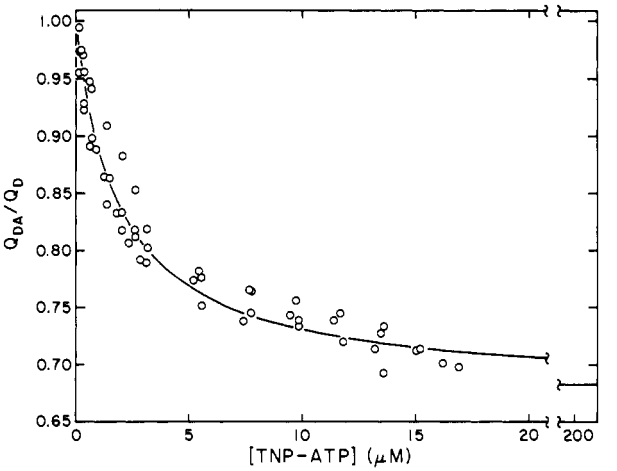

FIGURE 4: Titration of nucleotide site 3 on CF_1 containing CPM–ε with TNP-ATP. Q_D and Q_{DA} are the quantum yields of CPM in the absence and presence of TNP-ATP, respectively (387-nm excitation, 465-nm emission). The quenching data are plotted as a function of the total concentration of TNP-ATP. Prior to the titration, the CPM-labeled CF_1 was preequilibrated with TNP-ATP at nucleotide sites 1 and 2. All titrations were performed in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 6 mM $MgCl_2$, and 2 mM EDTA at 25 °C. The curve is a nonlinear least-squares fit of the data to eq 5 as described in the text.

were typically between 0.4 and 0.6 mol of CPM/mol of CF_1.

Stoichiometric amounts of TNP-ATP could be readily incorporated into nucleotide site 1 on CF_1 containing the CPM-labeled ε. The extent of energy transfer between these two sites was measured by comparing the fluorescence of the CPM-labeled CF_1 containing TNP-ATP bound to site 1 to that containing no TNP-ATP. After normalization with respect to protein concentrations, this measurement yielded an energy transfer efficiency of 0.12. This efficiency corresponds to a distance of 62 Å between the ε-sulfhydryl and nucleotide site 1.

Under the labeling conditions used, nucleotide site 1 exhibited a high preferential affinity for TNP-ATP over ATP. For this reason, the addition of excess ATP and monitoring of the subsequent increase in the CPM fluorescence upon displacement of TNP-ATP from nucleotide site 1 could not be used to obtain an alternative measure of the energy transfer between the two sites. However, this high preferential affinity did permit the converse experiment in which a small amount of TNP-ATP could be added to the CPM-labeled CF_1 such that a complete exchange of TNP-ATP for nucleotide at site 1 occurred with negligible binding of TNP-ATP at nucleotide sites 2 and 3. The extent of energy transfer between the CPM-labeled ε-sulfhydryl and TNP-ATP at nucleotide site 1 could then be obtained by monitoring the decrease of the CPM fluorescence upon incorporation of TNP-ATP. This measurement again gave a transfer efficiency of 0.12, which corresponds to a distance of 62 Å between the two sites.

In the presence of Mg^{2+}, stoichiometric amounts of tightly bound TNP-ATP could be incorporated into both nucleotide sites 1 and 2 on the CPM-labeled CF_1, with a total binding stoichiometry of 2 mol of TNP-ATP/mol of CF_1. By comparison of the fluorescence of this sample with that containing no TNP-ATP, a combined energy transfer efficiency of 0.19 between CPM at the ε-sulfhydryl and TNP-ATP at nucleotide sites 1 and 2 was obtained. With eq 3 and the distance calculated above for site 1, this efficiency corresponds to a distance of 66 Å between the ε-sulfhydryl and nucleotide site 2.

The measurement of energy transfer between the fluorescent-labeled ε-sulfhydryl and nucleotide site 3 was performed by titrating the CPM-modified enzyme with TNP-ATP and monitoring the decrease of the CPM fluorescence. To ensure that the observed quenching was due solely to binding at nucleotide site 3, the enzyme was first saturated with TNP-ATP at nucleotide sites 1 and 2. The results of this titration are shown in Figure 4 as a plot of the quantum yield ratios

for CPM vs. the total concentration of TNP-ATP. These data were fit to the equation

$$Q_{DA}/Q_D = 1 - E[EL]/[E_0] \quad (5)$$

where E is the energy transfer efficiency upon binding at site 3, [EL] is the concentration of bound ligand, $[E_0]$ is the total concentration of enzyme, and binding to a single site has been assumed. The concentration of EL can be readily calculated from the dissociation constant, K_d, for the enzyme–ligand complex, the total concentration of ligand, and the total enzyme concentration (Snyder & Hammes, 1984). A nonlinear least-squares fit of the data gave $K_d = 1.9 \pm 0.2$ μM and $E = 0.32 \pm 0.01$. This dissociation constant is well within the range of previously determined values for the dissociation of nucleotides from site 3 (Cantley & Hammes, 1975; Bruist & Hammes, 1981; Cerione & Hammes, 1982; Snyder & Hammes, 1984, 1985). The transfer efficiency obtained from the TNP-ATP titration can be used to calculate a distance of 49 Å between the ϵ-sulfhydryl and nucleotide site 3.

To measure the distance between the ϵ-sulfhydryl and the reduced γ-disulfide, the reduced disulfide of CF_1 containing CPM-labeled ϵ was modified with the acceptor species fluoresceinylmaleimide. The spectral overlap and energy transfer parameters for this donor–acceptor pair are summarized in Figure 3A and Table I, respectively. The extent of energy transfer between these two sites was obtained by measuring the fluorescence of CPM on the ϵ polypeptide in the presence and absence of fluoresceinyl maleimide at the reduced disulfide. An energy transfer efficiency of 0.73 was found for 0.81 mol of fluoresceinylmaleimide/mol of CF_1. This result indicates that the ϵ- and γ-disulfide sulfhydryls are close to one another; however, given the possible statistics for labeling the individual sulfhydryls of the γ-disulfide [cf. Snyder & Hammes (1985)] and the large R_0 for this donor–acceptor pair (50.6 Å), the observed transfer efficiency can be used to calculate only an upper limit of ≤35 Å for the distance between the two sites.

To obtain a more accurate measure of the separation between the ϵ-sulfhydryl and γ-disulfide, the energy transfer measurements described above were repeated with pyrenylmaleimide at the ϵ-sulfhydryl as an energy donor and CPM at the reduced γ-disulfide as an energy acceptor (see Figure 3B and Table I for the spectral overlap and energy transfer parameters). The shorter R_0 (31.2 Å) for this donor–acceptor pair enhances the sensitivity of the distance measurement for probes in close proximity to one another. Here again, the labeling stoichiometry at the ϵ-sulfhydryl was between 0.4 and 0.6 mol of donor/mol of CF_1. To eliminate uncertainties in the calculated distance due to uncertainties in labeling statistics of the sulfhydryls derived from the reduced disulfide, the reduced disulfide was fully saturated with CPM to a level of 2 mol of CPM/mol of CF_1. Comparison of the fluorescence of the pyrenylmaleimide-modified CF_1 with and without CPM at the reduced disulfide gave a transfer efficiency of 0.93. With the quenching relationships defined in eq 3, this efficiency corresponds to a distance of 23 Å between the two sites.

The ability to remove the ϵ polypeptide from CF_1 and the long lifetime of the pyrenylmaleimide donor made possible two further checks of the extent of energy transfer between the ϵ-sulfhydryl and the reduced γ-disulfide. In the first experiment, ethanol and glycerol were added to 20% (v/v) and 30% (v/v), respectively, to a solution containing the double-labeled CF_1, and the increase in the pyrenylmaleimide fluorescence upon the release of the ϵ polypeptide from CF_1 was monitored. After correction for dilution, a 10-fold increase in the pyrenylmaleimide fluorescence was observed. No increase in fluorescence was seen for a similarly treated CF_1 sample containing no CPM at the reduced disulfide. The observed energy transfer efficiency (0.90) was essentially the same as that obtained previously. In the second experiment, time-resolved fluorescence measurements were used to compare the lifetime of the pyrenylmaleimide fluorescence at the ϵ-sulfhydryl in the absence and presence of CPM at the reduced disulfide. In the absence of CPM, a two-component decay process was observed, with lifetimes of 17 and 68 ns. With CPM at the disulfide site, no long-lifetime decay components (>4 ns) were seen, in good agreement with the nearly complete quenching observed with the steady-state measurements.

To preserve sensitivity for the measurement of distances <30 Å, the energy transfer measurements between the ϵ-sulfhydryl and the dark- and light-site sulfhydryls of the γ subunit were again performed by using the pyrenylmaleimide–CPM donor–acceptor pair. For these experiments, however, the ϵ-sulfhydryl was labeled with CPM to serve as an energy acceptor for pyrenylmaleimide at the dark- and light-site sulfhydryls. The energy transfer parameters for these donor–acceptor pairs are summarized in Table I. Binding stoichiometries for the γ-sulfhydryls were approximately 0.7 and 0.4 mol of pyrenylmaleimide/mol of CF_1 at the dark and light sites, respectively. The CPM-labeled ϵ polypeptide was incorporated into the pyrenylmaleimide-labeled CF_1, and the fluorescence of pyrenylmaleimide in the absence or presence of the CPM acceptor was measured. For the dark-site sulfhydryl, the ATPase activity of the double-labeled sample was >90% inhibited relative to the ϵ-deficient sample containing only the donor label. The observed energy transfer efficiency for this donor–acceptor pair was 0.89/mol of CPM, corresponding to a distance of 26 Å between the ϵ-sulfhydryl and the dark-site γ-sulfhydryl.

When the light-site sulfhydryl of ϵ-deficient enzyme was labeled with pyrenylmaleimide, reconstitution with CPM-labeled ϵ polypeptide was greatly inhibited. In several separate experiments, the sum of the fraction of light-site sulfhydryl labeled and of labeled ϵ reconstituted could never be made greater than 1. Furthermore, no energy transfer was observed in these experiments. Since the light- and dark-site sulfhydryl groups are close to each other (Weiss & McCarty, 1977; Snyder & Hammes, 1985), the most reasonable interpretation of these results is that pyrenylmaleimide at the light-site sulfhydryl prevents the binding of CPM-labeled ϵ subunit to ϵ-deficient CF_1. Thus, the distance between the light-site sulfhydryl and the ϵ-sulfhydryl could not be obtained through energy transfer measurements.

Effect of ϵ Removal on Properties of the γ Subunit. The latent Ca^{2+}-dependent ATPase activity of CF_1 may be activated by one of several methods including treatment with heat, detergents or alcohols, proteases, and thiol reagents such as dithiothreitol [cf. McCarty & Moroney (1985)]. Heat, detergents, and alcohol all affect the removal of ϵ from CF_1 (Nelson et al., 1982; Holowka & Hammes, 1977; Patrie & McCarty, 1984; Richter et al., 1984; Yu & McCarty, 1985). Thiol activation results from the reduction of the disulfide bond in the γ polypeptide (Nalin & McCarty, 1984; Arana & Vallejos, 1982), while protease activation has been correlated with digestion of the α or γ polypeptides (Moroney & McCarty, 1982).

Both dithiothreitol and trypsin treatments of ϵ-deficient CF_1 caused an additional activation (approximately 2-fold) of the Ca^{2+}-ATPase activity (Figure 5). The half-time for the thiol activation of $CF_1(-\epsilon)$ (approximately 1.5 min) was more than 1 order of magnitude faster than for the normal enzyme

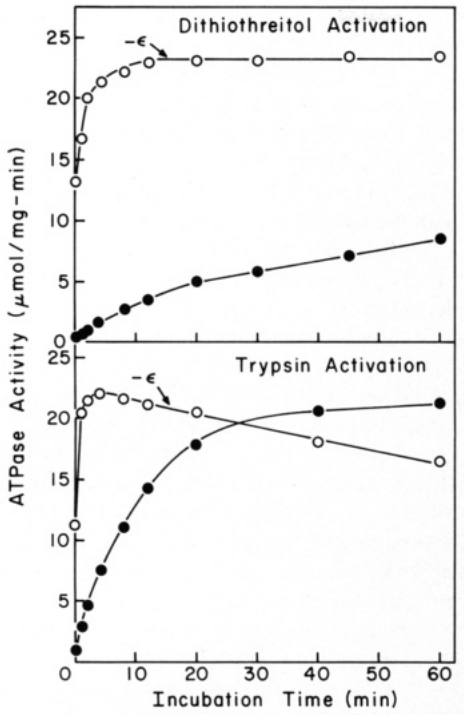

FIGURE 5: Activation of the Ca^{2+}-ATPase activities of CF_1 and $CF_1(-\epsilon)$ by dithiothreitol or trypsin. Incubation mixtures contained 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM ATP, 0.5 mg/mL CF_1 or $CF_1(-\epsilon)$, and (top panel) 20 mM dithiothreitol or (bottom panel) 10 μg/mL trypsin. Samples were assayed for Ca^{2+}-ATPase activity at the times indicated. Samples from the bottom panel were treated with soybean trypsin inhibitor (40 μg/μg of trypsin) before assay.

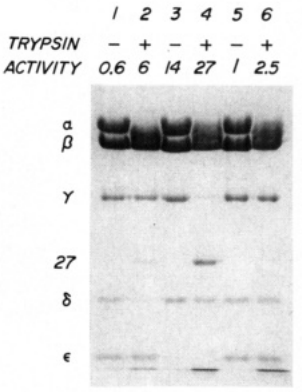

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of trypsin-treated CF_1, $CF_1(-\epsilon)$, and $CF_1(-\epsilon)$ reconstituted with ϵ. Trypsin (2 μg/100 μg of enzyme) was added where indicated, and samples were incubated for 5 min at room temperature. Phenylmethanesulfonyl fluoride (20 μg/μg of trypsin) was added to stop digestion. Aliquots were taken for Ca^{2+}-ATPase activity (micromoles per minute per milligram of protein) assay, and the remainder was subjected to electrophoresis on 12% polyacrylamide gels stained with Coomassie blue. Lanes 1 and 2, CF_1; lanes 3 and 4, $CF_1(-\epsilon)$; lanes 5 and 6, $CF_1(-\epsilon)$ reconstituted with ϵ. Each lane contained approximately 35 μg of enzyme preparation. The number 27 stands for the approximate molecular weight ($\times 10^{-3}$) of the major trypsin cleavage product of γ.

(Figure 5, top panel), suggesting a much greater accessibility of the reagent to the γ-disulfide in the absence of ϵ. Treatment of latent CF_1 with low amounts of trypsin induces Ca^{2+}-ATPase activity which approaches maximum activation after about 60 min of incubation (Figure 5, bottom panel). The half-time for this process varies between 10 and 20 min and has been correlated with cleavage of the α polypeptide of CF_1 (Moroney & McCarty, 1982). Removal of ϵ results in a rapid response of activity to trypsin with a half-time of approximately 1 min (Figure 5, bottom panel). Maximum activation under these conditions is reached within 3 or 4 min; activity gradually decreases with further incubation. This rapid additional activation by trypsin coincides with cleavage of the γ polypeptide apparently at a site which becomes exposed as a result of ϵ removal (Figure 6, lane 4). Reconstitution of $CF_1(-\epsilon)$ with the ϵ polypeptide returns the enzyme to the latent state and prevents the rapid activation by either dithiothreitol or trypsin and also prevents the hypersensitivity of the γ subunit toward tryptic cleavage (Figure 6, lane 6). Note that the α subunit is cleaved to a similar extent in CF_1, $CF_1(-\epsilon)$, and reconstituted CF_1 (Figure 6, lanes 2, 4, and 6), indicating that α cleavage is not involved in the rapid activation process.

Discussion

A stoichiometry of two ϵ polypeptides per CF_1 has been reported by several laboratories (Ravizzini et al., 1980; Binder et al., 1978; Béliveau et al., 1982; Nelson, 1976), whereas one ϵ per CF_1 was reported by Süss & Schmidt (1982). Our results indicate that a maximum of one ϵ binds tightly to ϵ-deficient CF_1. Moreover, only one ϵ is required for complete inhibition of the ATPase activity induced by ϵ removal. Since inhibition of ATPase activity is closely correlated with reinstatement of the ATP-synthesizing capacity of CF_1 (Richter et al., 1984), this suggests that only one ϵ is required for full functional activity of membrane-bound CF_1. The discrepancy between ϵ stoichiometry estimates may be explained, at least in part, by contamination of CF_1 preparations with small amounts of ribulosebisphosphate carboxylase. This enzyme interferes with sulfhydryl or tryptophan determinations which have been the basis for most ϵ stoichiometry estimates [cf. McCarty & Moroney (1985)]. The possibility that partial losses of ϵ during CF_1 preparation have contributed significantly to the difference in estimates is unlikely given the high apparent affinity of ϵ for CF_1, the relatively harsh conditions required for ϵ removal from CF_1, and the fact that the Ca^{2+}-ATPase activity of freshly purified CF_1 is usually less than 5% of that induced by ϵ removal.

Energy transfer distance measurements between the ϵ sulfhydryl and other groups on CF_1 all converged (within ±5%) to a single, unique locus. This is depicted in Figure 7. The spatial relationships between the three nucleotide binding sites, the dark- and light-accessible γ-sulfhydryls, the γ-disulfide, the Lucifer yellow reactive α-polypeptide site, and the membrane surface have been described elsewhere (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Nalin et al., 1985). The model shown was obtained by a least-squares refinement of all of the available energy transfer data as described in the Appendix. The ϵ-sulfhydryl is located in the lower half of the molecule, approximately equidistant from the "dark" and disulfide sulfhydryls of the γ subunit and well removed from all three nucleotide binding sites. The distance between the ϵ-sulfhydryl and the site on one of the α subunits specifically labeled by the probe "Lucifer yellow" was reported previously (Nalin et al., 1985). This distance is consistent with the results presented here. An estimate of 40 Å was made for the distance between the ϵ-sulfhydryl and the membrane surface from the model. The errors inherent in these distance measurements have been previously discussed (Snyder & Hammes, 1985).

If a compact, globular structure for the ϵ polypeptide is assumed, the model predicts a very close association between ϵ and the sulfhydryl-containing domains of the γ subunit. This may explain our inability to measure energy transfer between ϵ and the light-accessible γ-sulfhydryl if, for example, steric hindrance prevented labeled ϵ binding to the light-site modified

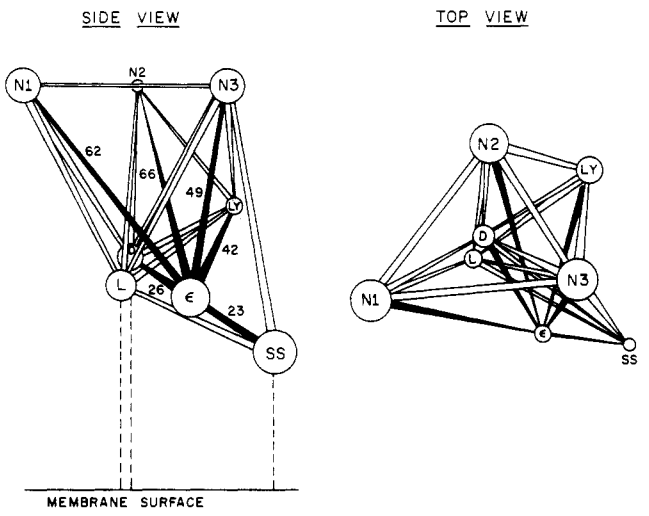

FIGURE 7: Schematic illustration of the spatial relationships among the nucleotide binding sites, the sulfhydryl-containing residues of the γ and ϵ polypeptides, the Lucifer yellow reactive site of an α polypeptide, and the membrane surface, for the intact coupling factor complex as determined through fluorescence resonance energy transfer measurements (see Appendix). N1–N3 are nucleotide sites 1–3; D, L, and SS are the "dark", "light", and disulfide sulfhydryls of the γ polypeptide, respectively; ϵ is the sulfhydryl site of the ϵ polypeptide; LY is the Lucifer yellow site. Views perpendicular and parallel to the bilayer surface are shown with the size of the balls corresponding to the depth of field for the various sites. The lines are site–site separations obtained in this and other work (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Nalin et al., 1985). The distances measured relative to the ϵ-sulfhydryl site on CF_1 are given in angstroms. The positions of the essential tyrosine residues of the β subunit of CF_1 (not shown) are not uniquely determined by the fluorescence energy transfer measurements; however, they can be approximately located in a plane parallel to, and 30 Å below, the plane defined by the three nucleotide binding sites with each β-tyrosine located directly below a corresponding nucleotide binding site (Snyder & Hammes, 1985). The distances to the membrane are maximum separations since the data do not require that the lines be perpendicular to the membrane.

enzyme. A distance of 22 Å is predicted between these two sulfhydryls (Figure 7). A marked feature of the structural model in Figure 7 is the asymmetry of CF_1; in particular, the nucleotide binding sites are nonequivalent with respect to both the ϵ and γ polypeptides. Whether or not the nucleotide sites are functionally asymmetric is a matter of considerable controversy [cf. Bruist & Hammes (1982) and Kohlbrenner & Boyer (1983)].

Removal of ϵ strongly influenced the reactivity of γ toward thiol reduction and proteolytic cleavage. Similar results were obtained for heat-treated CF_1 (Nalin & McCarty, 1984; Moroney & McCarty, 1982). Our results clearly establish the role of ϵ in this process. In view of the close physical association between ϵ and γ indicated by energy transfer measurements, the simplest interpretation of this effect is that ϵ binds to, or sufficiently close to, γ such that it masks the reactive groups on the γ polypeptide. An earlier finding (Baird & Hammes, 1976) that cross-linking reagents cause the formation of $\epsilon\gamma$ aggregates supports this interpretation.

Alterations in the structure of the γ subunit of CF_1 by disulfide reduction, specific group modification, or proteolytic cleavage all induce marked changes in the catalytic properties of CF_1 either in its membrane-bound or in its soluble form (Ketcham et al., 1984; Moroney & McCarty, 1982). Such effects have formed the basis for the proposal (Nelson, 1981; McCarty & Moroney, 1985) that the γ subunit plays a central role in regulating the catalytic activity of the enzyme. Our results are consistent with this concept in that they suggest that the effect of the ϵ polypeptide on CF_1 activation may also be mediated by the γ subunit. Convincing evidence for this was provided by an additional observation[2] that ϵ is rendered completely ineffective as an ATPase inhibitor after tryptic cleavage at a specific site on the γ subunit. Proteolytic cleavage of the γ subunit of *E. coli* F_1 also prevents inhibition of the ATPase activity by added ϵ (Smith & Sternweis, 1977). Studies with the isolated polypeptides of *E. coli* coupling factor 1 further indicate that the binding site for ϵ is located exclusively on the γ subunit (Larson & Smith, 1977; Dunn, 1980).

The physiological significance of ϵ as an ATPase inhibitor was recently questioned (Richter et al., 1984). The conditions which promote ATP hydrolysis by CF_1 on the membrane also promote ATP synthesis (Mills & Mitchell, 1982; Ketcham et al., 1984). Since there is a stringent requirement for ϵ during ATP synthesis (Richter et al., 1984), it is improbable that ϵ dissociates under these conditions. The very high apparent affinity of CF_1 for ϵ strengthens this argument. However, energy-dependent conversion of CF_1 to active forms may be associated with alterations in ϵ–γ interactions. There is some indication that this is the case. Illumination of thylakoid membranes causes a marked increase in the sensitivity of the γ subunit to cleavage by trypsin (Moroney & McCarty, 1982) which is very similar to that elicited by removal of ϵ. Thus, at least with respect to a trypsin-sensitive region of the γ subunit, the conformations of membrane-bound CF_1 in an active form and CF_1 deficient in ϵ are similar. Further work on ϵ–γ interactions may, therefore, give insights into the intriguing question of the mechanism of activation of membrane-bound CF_1.

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APPENDIX

Three-Dimensional Modeling of CF_1 Spatial Relationships. With the results presented in this paper, over 30 distances have now been measured between specific sites within the chloroplast coupling factor by use of fluorescence resonance energy transfer techniques (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Nalin et al., 1985). These sites include the three nucleotide binding sites of CF_1, the sulfhydryl-containing residues of the γ and ϵ polypeptides, the Lucifer yellow reactive site of an α polypeptide, and the membrane surface of the reconstituted system. The measured distances provide sufficient information such that the relative locations of the various sites on CF_1 can be fixed with respect to one another. Also, distance measurements performed with the reconstituted system permit the determination of the approximate orientation of these sites relative to the membrane surface in the intact coupling factor. Three-dimensional modeling can be used to show that the measured distances are consistent within ~5–10% with the assumption that the orientation of transition dipoles is isotropically averaged. Indeed, these results indicate that the possible errors arising from the choice of the isotropic average for κ^2 ($^2/_3$) are no more significant than the expected experimental uncertainties associated with energy transfer measurements.

[2] M. L. Richter, unpublished observation.

To perform a rigorous determination of the location of the labeling sites on CF_1, given the available distance information, a hand-built structural model was used to obtain initial estimates of the three-dimensional coordinates of each site. By allowing these coordinates to vary, a simplex algorithm (Nelson & Mead, 1965) was then used to minimize the "Chi square" deviation between the measured and fitted distances. To fix the single rotational degree of freedom relative to the membrane surface, the three nucleotide binding sites of CF_1 were assumed to be equidistant from the membrane. This assumption places no constraints on the fitting procedure with regard to the relative positions of the sites within CF_1 but does fix their orientation relative to the membrane surface. In the "best-fit" model, the maximum deviation between the measured and fitted distances was ≤3%. Figure 7 illustrates the best-fit model.

Registry No. ATP synthetase, 37205-63-3; ATPase, 9000-83-3.

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