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Structural Organization of Chloroplast Coupling Factor†

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Received October 25, 1984

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

† This work was supported by a grant from the National Institutes of Health (GM 13292).
‡ National Institutes of Health Postdoctoral Fellow (GM 09061).

[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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of this disulfide results in a partial activation of ATP hydrolysis (Nalin & McCarty, 1984). This activity can be abolished by reacting NBD-Cl with a specific tyrosine residue on the β-polypeptides (Deters et al., 1975; Cantley & Hammes, 1975).

Previously, fluorescence resonance energy transfer techniques, with nucleotide analogues and fluorescent maleimide derivatives as labeling reagents, have been used to investigate the spatial relationships between the light-site sulfhydryl, the reduced disulfide, and the nucleotide binding sites of CF_1 (Cerione & Hammes, 1982; Snyder & Hammes, 1984). In addition, the distances of the γ-sulfhydryl sites from the membrane surface for the intact enzyme have been obtained by measuring the extent of energy transfer between fluorescent maleimide derivatives covalently attached to specific γ-sulfhydryl sites and acceptor fluorophores uniformly distributed along the membrane surface (Cerione et al., 1983; Snyder & Hammes, 1984). The results of these studies have been used to create a working model for the overall structural characteristics of the coupling factor complex (Snyder & Hammes, 1984). In the present study, fluorescence energy transfer measurements are used to obtain spatial maps, consistent with this structural model, for the dark-site γ-sulfhydryl and the β-tyrosines that react with NBD-Cl. The results provide insight into the structural organization of the α-, β-, and γ-polypeptides in CF_1.

Materials and Methods

Chemicals. ATP (vanadium free) and Triton X-100 were from Sigma Chemical Co. TNP-ATP and all fluorescent maleimide derivatives were from Molecular Probes, Inc. NBD-Cl was obtained from Pierce Chemical Co. and quinine sulfate from Aldrich Chemical Co. Hydroxylapatite (fast flow) was from Calbiochem-Behring. All other chemicals were high-quality commercial grades, and all solutions were prepared from deionized, distilled water.

CF_1 Preparation. CF_1 was prepared from fresh market spinach by known procedures (Lien & Racker, 1971; Binder et al., 1978). The purified enzyme was stored as a precipitate in 2 M ammonium sulfate, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.5 mM ATP and kept at 4 °C for periods no longer than 1 month. The molar concentrations of CF_1 were determined by using 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). The enzyme was assayed as previously described after activation for 4 min at 63 °C in 7 mM dithiothreitol, 40 mM Tris-HCl (pH 8.0), 40 mM ATP, and 2 mM EDTA (Moroney et al., 1983) and had a specific activity typically in the range of 15–20 μmol/(mg·min) at 25 °C.

ϵ-Deficient CF_1 was prepared by hydroxylapatite chromatography of heat-treated CF_1 (Patrie & McCarty, 1984). One gram of dehydrated hydroxylapatite was suspended in 40 mL of 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 15 mM sodium phosphate (pH 8.0), and 1 mM ATP (column buffer), and the fine particles were decanted. The hydroxylapatite was resuspended, and the fine particles were decanted twice more. A 0.5 (i.d.) × 4 cm Pasteur pipet column was poured and washed with 5 mL of column buffer. One milligram of CF_1 was heated at 63 °C for 4 min in 0.3 mL of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 40 mM ATP, and 0.04% Triton X-100 and after being cooled to room temperature was applied to the hydroxylapatite column. The enzyme was eluted with 1.5 mL of column buffer. The solution eluted from the column was dialyzed against 1000 volumes of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5 mM ATP for at least 12 h and the protein concentrated through ammonium sulfate precipitation. The preparation was determined to be >95% ϵ deficient as visualized in sodium dodecyl sulfate gel electrophoresis patterns (Chua, 1980).

Chemical Modification of CF_1. Specific modification of the exposed γ-sulfhydryl (dark site) on ϵ-deficient CF_1 was obtained by incubating the enzyme (2–5 μM) with either 50 μM CPM or 25 μM pyrenylmaleimide for 2–10 min in 10 mM Tris-HCl, 50 mM NaCl, and 2 mM EDTA (labeling buffer). Unreacted probe was removed by passing the solution through two consecutive 3-mL centrifuge columns containing Sephadex G-50 equilibrated with the labeling buffer (Penefsky, 1977). Labeling of residual ϵ subunit was negligible as visualized by the fluorescence of sodium dodecyl sulfate slab gels illuminated with near-ultraviolet light. Some labeling of the α and β subunits of CF_1 was usually observed for the CPM-modified enzyme, presumably due to aminolysis of hydrolyzed maleimide rings (Wu et al., 1976); however, this nonspecific contaminant was always less than 5% of the total label present on the γ subunit.

The γ-disulfide site on ϵ-deficient CF_1 was covalently labeled by first incubating the enzyme (2–5 μM) with 2 mM *N*-ethylmaleimide for 15 min in labeling buffer to block exposed sulfhydryls. Dithiothreitol was then added to 10 mM and reacted for 1 h to reduce the γ-disulfide. In contrast to latent CF_1, the γ-disulfide on the heat-treated enzyme is readily accessible to reduction by dithiothreitol (Nalin & McCarty, 1984). The excess dithiothreitol was removed by passing the enzyme through two consecutive centrifuge columns equilibrated with the labeling buffer. Finally, fluoresceinylmaleimide was added to 50 μM and the solution incubated for 5 min before removal of the unreacted probe by column centrifugation. Labeling of the buried γ-sulfhydryl of CF_1 (light site) on energized thylakoids with pyrenylmaleimide and subsequent isolation of the solubilized CF_1 complex were performed as described previously (Cerione et al., 1983; Nalin et al., 1983). For both cases, only a single fluorescent band, corresponding to the γ subunit of CF_1, was observed after sodium dodecyl sulfate gel electrophoresis of the labeled enzyme.

Procedures for specific labeling of the tight nucleotide binding sites of CF_1 with TNP-ATP have been described elsewhere (Cerione & Hammes, 1982; Snyder & Hammes, 1984). Labeling of the β-tyrosines of CF_1 with NBD-Cl was performed by incubating the enzyme (1.0–1.5 μM) with 50 μM NBD-Cl in labeling buffer. The time course of the reaction was followed by monitoring the change in absorbance at 400 nm. The 400-nm absorbance of the NBD-CF_1 solution was corrected for the absorbance of an equal concentration of NBD-Cl in buffer (in the absence of protein) and an equal concentration of CF_1 (in the absence of NBD-Cl).

Probe concentrations and labeling stoichiometries for each probe were calculated by using 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), 2.51×10^4 M^{-1} cm^{-1} at 418 nm for bound TNP-ATP (Cerione & Hammes, 1982), and 1.07×10^4 M^{-1} cm^{-1} at 400 nm for NBD-Cl reacted at the β-tyrosine of CF_1 (Cantley & Hammes, 1975). For calculation of the stoichiometries, corrections were made for probe absorbance at 277 nm, CF_1 light scattering at all wavelengths, and probe–probe spectral overlaps.

Spectroscopic Measurements. Absorbance measurements were made with a Cary 118 spectrophotometer. Steady-state

Table I: Energy Transfer Parameters for CF_1[a]

donor	location	fluorescence max (nm)	Q_D	P^b	acceptor	location	absorbance max (nm)	R_0 (Å)[c]
CPM	dark	470	0.78	0.38	TNP-ATP	N_1–N_3	418/480	45.6
CPM	dark	470	0.78	0.38	FM	DiSH	495	51.4
PM	light	375/395	0.30	0.25	NBD	β-Tyr	390	32.0
PM	dark	375/395	0.30	0.24	NBD	β-Tyr	390	32.0

[a] Abbreviations: dark and light, dark- and light-site γ-sulfhydryls of CF_1, respectively; N_1–N_3, nucleotide binding sites 1–3 on CF_1; DiSH, γ-disulfide site; β-Tyr, NBD-reactive β-tyrosine residues; PM, pyrenylmaleimide; FM, fluoresceinylmaleimide. [b] Steady-state polarization measured at fluorescence excitation and emission maxima. [c] Calculated with eq 3 and the spectral properties of the donor and acceptor species. The value of κ^2 is assumed to be $^2/_3$.

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 (Cerione & Hammes, 1982). Quantum yields for the fluorescent-labeled CF_1 samples were calculated by a comparative method according to Parker & Reese (1966). Quinine sulfate in 0.1 N H_2SO_4 was used as the fluorescence standard and was assumed to have a quantum yield of 0.70 (Scott et al., 1970).

Fluorescence Resonance Energy Transfer Measurements. For all CF_1 energy transfer determinations, the donor maleimide fluorescence was corrected for the measured probe absorbance, protein light scattering, and stoichiometries of acceptor binding. Efficiencies (E) of energy transfer were obtained from the relationship

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

where Q_{DA} and Q_D are the donor quantum yields in the presence and absence of acceptor, respectively. All reported transfer efficiencies were the average of at least two measurements, with standard deviations of ±0–4% of the average value. Distances between specific donor and acceptor sites were calculated by using

$$E = \sum_{i=1}^{N_A}(R_0/R_i)^6/[1 + \sum_{i=1}^{N_A}(R_0/R_i)^6] \quad (2)$$

where N_A is the number of acceptors contributing to the quenching of a single donor, R_i is the distance between a specific donor and acceptor pair, and R_0 is the distance at which the energy transfer efficiency is 0.5 for a single donor–acceptor pair. Equation 2 assumes each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of CF_1. When the nucleotide binding sites of CF_1 were used as acceptor sites, either a single donor–acceptor pair was present or all but one of the donor–acceptor distances had previously been measured such that eq 2 contained only one unknown. For the case of the γ-disulfide, the distances to each individual sulfhydryl were assumed to be equal (Snyder & Hammes, 1984). A detailed discussion of the analysis of the observed energy transfer for multiple NBD acceptor sites will be presented later. In eq 2, R_0 is given by (Förster, 1959)

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

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. Upper and lower bounds for κ^2 can be calculated from the observed polarization of donor and acceptor molecules (Dale et al., 1979). 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.

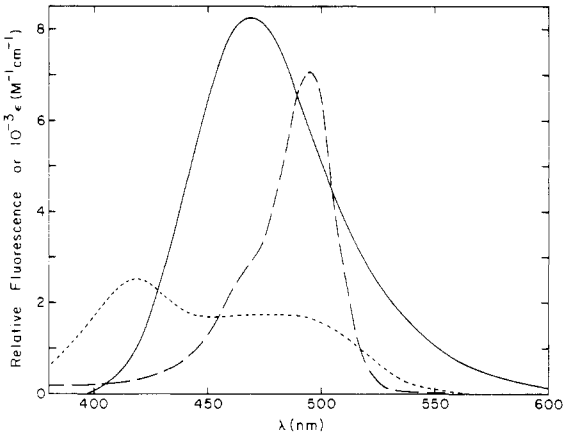

FIGURE 1: Spectral overlap of the corrected fluorescence emission for CPM (—) covalently bound to the dark-site γ-sulfhydryl with the extinction coefficient (ε) of fluoresceinylmaleimide (···) bound to the γ-disulfide site and TNP-ATP (- - -) bound at the nucleotide binding sites of CF_1. The fluorescence excitation for the CPM-modified CF_1 was 387 nm. All spectra were taken in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA at 25 °C.

RESULTS

Mapping of CF_1 Dark-Site γ-Sulfhydryl. In order to perform resonance energy transfer measurements with the dark site of the γ subunit, this sulfhydryl was covalently modified with CPM. The use of the ε-deficient enzyme prevents labeling of the sulfhydryl of the ε subunit. The fluorescence properties of CPM when attached to the dark site are summarized in Table I. Its high quantum yield is similar to that for CPM at the other γ-sulfhydryl sites (Snyder & Hammes, 1984). Its high polarization, indicative of a very restrictive environment, is in agreement with values obtained for other fluorescent maleimide derivatives reacted with the dark-site sulfhydryl (Cerione et al., 1983; Nalin et al., 1983; Nalin & McCarty, 1984). Under optimal conditions, labeling stoichiometries were generally between 0.6 and 0.8 mol of CPM/mol of CF_1. The fluorescence emission spectrum of the CPM has an excellent overlap with the absorbance spectrum of both TNP-ATP bound to the nucleotide sites of CF_1 and fluoresceinylmaleimide located at the γ-disulfide site (see Figure 1). The characteristic R_0 values for these donor–acceptor pairs are in the range 45–50 Å.

Nucleotide site 1 on the ε-deficient enzyme could be readily labeled with TNP-ATP. Calculated labeling stoichiometries were in slight excess of 1 mol of TNP-ATP/mol of CF_1, probably due to the overestimation of the molecular weight of CF_1 with ε removal and the loss of some δ subunit. After the dark site was labeled with CPM, TNP-ATP was incorporated into nucleotide site 1. The efficiency of energy transfer between the labeled dark-site sulfhydryl and nucleotide site 1 was then measured by comparing the fluorescence for the CF_1-containing TNP-ATP to that containing no TNP-ATP, normalizing with respect to the protein concentrations. The measurement yielded a transfer efficiency of 0.41/mol of

bound TNP-ATP. This efficiency corresponds to a distance of 48 Å between the dark-site γ-sulfhydryl and nucleotide site 1.

In an attempt to obtain a second measure of the transfer efficiency between CPM at the dark site and TNP-ATP at nucleotide site 1, excess ATP was added to the enzyme containing TNP-ATP bound to nucleotide site 1, and the increase in fluorescence caused by a displacement of the TNP-ATP was monitored. Unfortunately, in contrast with latent-intact enzyme (Snyder & Hammes, 1984), the exchange of ATP for TNP-ATP was found to be very slow, with site 1 exhibiting a high preferential affinity for TNP-ATP over ATP. However, after a 9-h incubation in the presence of a 20000-fold excess of ATP over CF_1, a minimum transfer efficiency of $E > 0.32$ between the two sites could be estimated. This value yields a maximum separation of <52 Å.

The tightness of TNP-ATP binding at nucleotide site 2 in the presence of Mg^{2+} was unaltered by ϵ removal. However, in the presence of medium nucleotide, a complete exchange of ATP for TNP-ATP on the ϵ-deficient enzyme occurred within a few hours. These results provided two methods for measuring the quenching of CPM at the dark site by TNP-ATP bound at site 2. Initially, TNP-ATP was incorporated into both nucleotide sites 1 and 2 on the CPM-modified enzyme, yielding binding stoichiometries of ~2 mol of TNP-ATP/mol of CF_1. The combined quenching of CPM by TNP-ATP bound at sites 1 and 2 was then measured by comparing the fluorescence with that of enzyme containing no TNP-ATP. This measurement gave a transfer efficiency of 0.68. By use of eq 2, the distance calculated above for site 1, and the combined transfer efficiency to sites 1 and 2, a separation of 43 Å between the dark-site γ-sulfhydryl and nucleotide site 2 can be calculated. In the alternative experiment, excess ATP was added to the enzyme containing TNP-ATP bound at both sites 1 and 2, and the increase in CPM fluorescence upon displacement of TNP-ATP bound at site 2 was monitored. After correction for the small amount of exchange at site 1 during the course of site 2 displacement, an 82% increase in CPM fluorescence was observed due to the removal of TNP-ATP bound at site 2. By use of eq 2 and the known transfer efficiency of 0.41 to site 1 alone, this fluorescence increase again yields a distance of 43 Å between the dark-site sulfhydryl and nucleotide site 2.

The measurement of energy transfer between the dark-site γ-sulfhydryl and nucleotide site 3 was performed by titrating the modified ϵ-deficient enzyme with TNP-ATP and monitoring the decrease in CPM fluorescence. To ensure that the observed quenching was due solely to binding at site 3, the enzyme was first saturated with TNP-ATP at nucleotide sites 1 and 2. This titration is shown in Figure 2 as a plot of the quantum yield ratios for CPM vs. the total concentration of TNP-ATP. The data were fit to the equation

$$Q_{DA}/Q_D = 1 - E[EL]/[E_0] \quad (4)$$

where E is the relative transfer efficiency upon binding at the site, [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 is a function of 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 $K_d = 1.7 \pm 0.2$ μM, well within the range of previously determined values for the binding of nucleotides at site 3 (Cantley & Hammes, 1975; Bruist & Hammes, 1981; Cerione & Hammes, 1982; Snyder & Hammes, 1984), and $E = 0.22 \pm 0.01$. The total transfer efficiency for all three

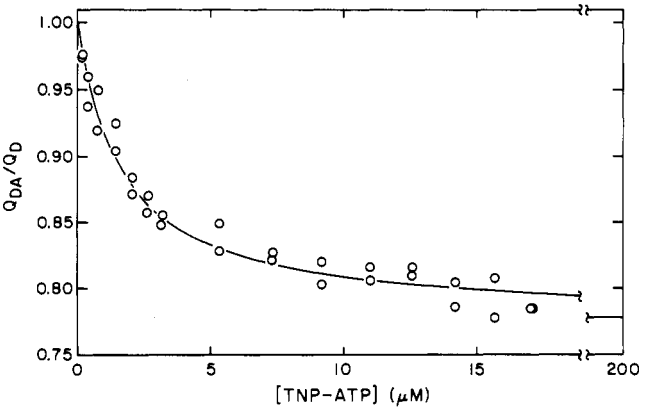

FIGURE 2: Plot of the fluorescence quenching of CPM bound to the dark-site γ-sulfhydryl of CF_1 vs. the total concentration of TNP-ATP. Here, Q_D and Q_{DA} are the quantum yields in the absence and presence of TNP-ATP bound to site 3, respectively (387-nm excitation, 465-nm emmission). CF_1–CPM was preequilibrated with TNP-ATP at sites 1 and 2 before the titration of site 3. 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 4 as described in the text.

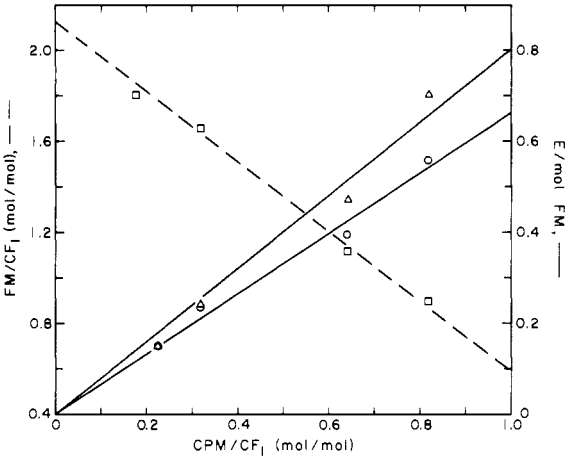

FIGURE 3: Plots of the acceptor labeling stoichiometry of fluoresceinylmaleimide at the γ-disulfide site (□) and the observed donor–acceptor transfer efficiencies, transformed into the values expected for one acceptor per CF_1 molecule, vs. the donor labeling stoichiometry of CPM at the dark-site γ-sulfhydryl. The transformation of the energy transfer data was performed as described in the text, assuming either random (△) or restricted (○) disulfide labeling. The lines are least-squares fits of the data. Extrapolation of the fitted transfer efficiencies to 1.0 mol of CPM/mol of CF_1 yields values of 0.80 and 0.67 per mole of fluoresceinylmaleimide for random and restricted disulfide labeling, respectively.

nucleotide sites containing 1 mol of bound TNP-ATP is thus 0.75. By use of the known distances to sites 1 and 2 and the quenching relationships defined in eq 2, this efficiency corresponds to a distance of 47 Å separating the dark-site γ-sulfhydryl and nucleotide site 3.

In order to calculate the distance separating the dark-site γ-sulfhydryl and the γ-disulfide site, the CPM (donor) modified CF_1 was treated with dithiothreitol to reduce the disulfide and then incubated with fluoresceinylmaleimide (acceptor) to label newly exposed free sulfhydryls. Prior to dithiothreitol modification, all other exposed sulfhydryls were blocked with *N*-ethylmaleimide. The presence of CPM at the dark site, however, inhibited the labeling of the γ-disulfide. This result is shown in Figure 3 as a plot of the stoichiometry of fluoresceinylmaleimide labeling vs. the fractional labeling by CPM of the dark sulfhydryl. At low CPM stoichiometries, high fluoresceinylmaleimide stoichiometries are observed,

approaching ~2 mol/mol of CF_1 in the absence of donor labeling. With increasing donor stoichiometries, a linear decrease in fluoresceinylmaleimide stoichiometry is observed, with CPM labeling of the dark-site sulfhydryl resulting in ~75% inhibition of acceptor labeling under the given experimental conditions. To correct for this nonrandom labeling (i.e., preferential acceptor labeling of enzyme molecules containing no donor label), the transfer efficiency for the CPM–fluoresceinylmaleimide pair, normalized with respect to the protein concentrations of donor- and donor–acceptor-labeled samples, was measured as a function of the donor stoichiometry. The observed efficiencies were then extrapolated to 1 mol of CPM/mol of CF_1. This procedure ensures that the final quenching value is representative of the intrinsic transfer efficiency between the labeled dark and disulfide sites. The results of these measurements are shown in Figure 3. The observed transfer efficiencies have been transformed into the values expected for one acceptor per CF_1 molecule. This transformation was performed for two limiting cases by using the quenching relationship in eq 2 and the measured acceptor stoichiometries. In the first, the modification of the two individual sulfhydryls of the disulfide site was assumed to be random such that the fraction of CF_1 molecules containing one, two, or no acceptor(s) was determined by the binomial distribution. In the second, comodification of both reduced sulfhydryls was assumed to be severely restricted such that for acceptor stoichiometries <1 mol/mol, all enzyme molecules contained only one acceptor label and for stoichiometries >1 mol/mol, at least one acceptor label. Both transformations yield a linear increase in transfer efficiency with donor stoichiometry and, as expected, exhibit an absence of energy transfer in the limit of negligible donor labeling. The extrapolated efficiencies at 1 mol of CPM/mol of CF_1, 0.80 and 0.67 per mole of fluoresceinylmaleimide, respectively, for random and restricted disulfide labeling, give a separation between the dark and disulfide sites in the range 41–46 Å. The steady-state polarization of fluoresceinylmaleimide at the γ-disulfide site for these measurements was 0.21.

Mapping of NBD-Cl-Reactive β-Tyrosines of CF_1. Previously, energy transfer techniques have been used to investigate the spatial relationships between the β-tyrosines that react with NBD-Cl and the γ-disulfide site (Cantley & Hammes, 1976) and nucleotide binding sites (Cerione & Hammes, 1982) of CF_1. In an attempt to gain a more complete understanding of the overall organization of the NBD sites on CF_1, the spatial relationships between these sites and the dark and light site labeled γ-sulfhydryls were investigated further. To perform these studies, each of the γ-sulfhydryls was separately modified with the fluorescent probe pyrenylmaleimide. The modification of the dark-site sulfhydryl was done with the ϵ-deficient enzyme to ensure specific labeling. Under the given conditions (see Materials and Methods), labeling stoichiometries were generally in the range 0.3–0.5 mol of pyrenylmaleimide/mol of CF_1. The fluorescence properties for pyrenylmaleimide at both the light- and dark-labeled γ-sulfhydryls are summarized in Table I. The characteristics of this probe at each sulfhydryl site are essentially equivalent. The spectral overlap of the pyrenylmaleimide fluorescence emission with the absorbance of NBD reacted at the β-tyrosine site is shown in Figure 4. This overlap yields a value for R_0 of 32.0 Å for the pyrenylmaleimide(donor)–NBD(acceptor) pair.

The time course of the reaction of NBD-Cl with CF_1 modified with pyrenylmaleimide at either the dark or the light site, monitored at 400 nm, is shown in Figure 5. Within 10

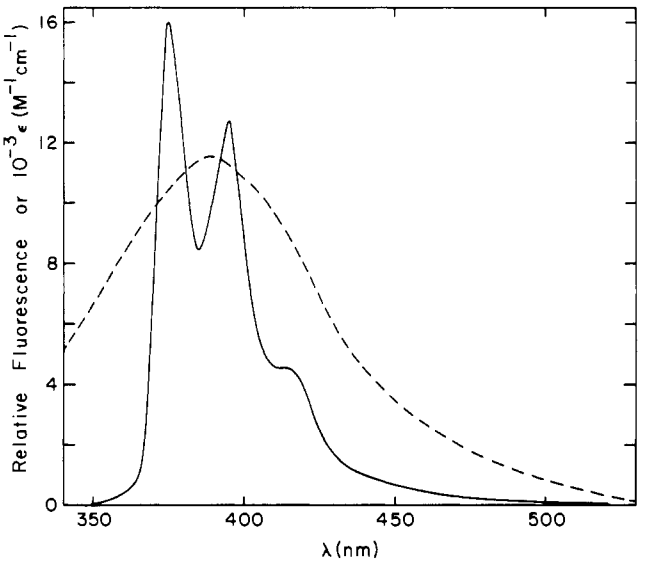

FIGURE 4: Spectral overlap of the corrected fluorescence emission for pyrenylmaleimide (—) covalently bound to either the light- or the dark-site γ-sulfhydryl with the extinction coefficient (ϵ) of NBD-Cl (---) reacted with the β-tyrosines of CF_1. The fluorescence excitation for pyrenylmaleimide-modified CF_1 was 343 nm. The pyrenylmaleimide spectra were taken in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA at 25 °C. For NBD-Cl, 6 mM $MgCl_2$ also was present.

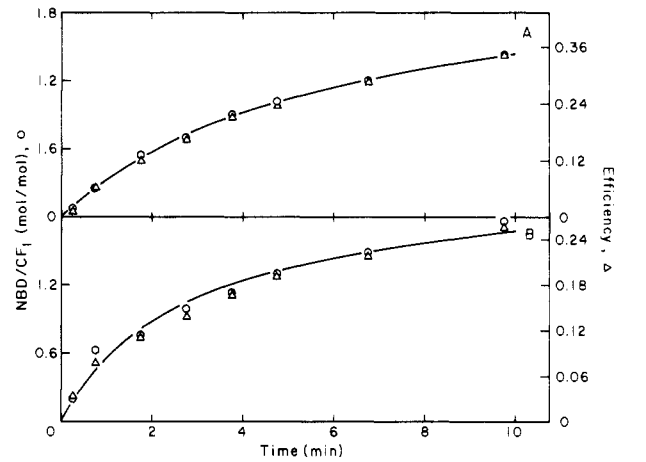

FIGURE 5: Time course of NBD (acceptor) labeling stoichiometry at the β-tyrosine sites (O) after addition of 50 μM NBD-Cl to CF_1 covalently modified with pyrenylmaleimide (donor) at either the light-site (A) or the dark-site (B) γ-sulfhydryl. The concentrations of CF_1 were 1.5 and 1.0 μM for the enzyme with light and dark sites labeled, respectively. Corresponding plots of the transfer efficiencies between pyrenylmaleimide and the bound NBD vs. the time of incubation with NBD-Cl are also shown (Δ). The curves are nonlinear least-squares fits of the binding data to eq 5 as described in the text.

min, labeling stoichiometries for NBD at the β-tyrosine sites approach 2 mol/mol of CF_1. In addition, the dark site labeled (ϵ-deficient) CF_1 appears to have a slightly higher reactivity than the light side labeled (intact) enzyme. The presence of pyrenylmaleimide at the light site did not alter the NBD reactivity relative to the unlabeled intact CF_1. The presence of 5 mM ATP in the incubation buffer, however, significantly reduced the rate, but not the extent, of NBD labeling. For incubation times >10 min, accurate stoichiometries were difficult to obtain due to the onset of nonspecific modification of exposed amino groups. This nonspecific binding could be monitored through changes in absorbance at wavelengths >400 nm (Deters et al., 1975). The rate of NBD binding at the two β-tyrosines was analyzed with the assumption that two con-

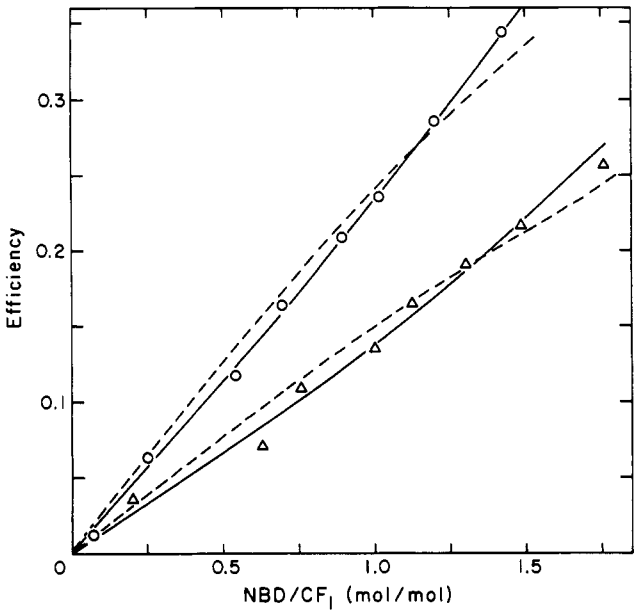

FIGURE 6: Plots of the observed transfer efficiency between pyrenylmaleimide covalently attached to the light-site (○) and dark-site (△) γ-sulfhydryls and NBD-Cl reacted at the β-tyrosine sites vs. the stoichiometry of NBD labeling. The curves are nonlinear least-squares fits of the data to eq 6 with (---) and without (—) the stipulation that $R_1 = R_2$.

secutive first-order reactions occur characterized by the first-order rate constants k_1 and k_2. In this case

$$\text{mol of NBD/mol of CF}_1 = 2 + [(k_1 - 2k_2)e^{-k_1 t} + k_1 e^{-k_2 t}]/(k_2 - k_1) \quad (5)$$

For both the light and dark site labeled enzyme, a nonlinear least-squares analysis of the data for incubation times <10 min gave best fits with modification of the second β-tyrosine occurring at a rate approximately one-fifth that of the first. This result is in good agreement with a previous kinetic analysis of NBD labeling at the β-tyrosine sites (Cantley & Hammes, 1975). The possible modification of a third β-tyrosine by NBD-Cl at longer times cannot be ruled out but is likely to proceed at a rate much slower than for the other sites that react with NBD-Cl.

The measurements of energy transfer between pyrenylmaleimide at the γ-sulfhydryl sites and NBD-Cl reacted with the β-tyrosines were performed by monitoring the time course of the decrease in pyrenylmaleimide fluorescence upon binding of NBD. The variation of the observed transfer efficiencies with time for both the light and dark site labeled CF_1 is presented in Figure 5. The time course of the efficiency follows closely that of the reaction of NBD-Cl with the β-tyrosine sites, which is also included in Figure 5. The result, together with the observation that pyrenylmaleimide at the γ-sulfhydryls does not affect either the rate or the extent of NBD labeling, suggests that preferential reaction of NBD-Cl with CF_1 molecules containing no donor label is not occurring, although pyrenylmaleimide stoichiometries are significantly less than 1 mol/mol of enzyme. Plots of the total transfer efficiency as a function of the NBD labeling stoichiometry are shown in Figure 6. For both cases, a roughly linear increase in transfer efficiency is observed with respect to the amount of NBD bound to the enzyme. Distances between the γ-sulfhydryl sites and the two β-tyrosine sites were obtained by fitting the energy transfer data to an equation of the form

$$E = F_1 \frac{(R_0/R_1)^6}{1 + (R_0/R_1)^6} + F_2 \frac{(R_0/R_1)^6 + (R_0/R_2)^6}{1 + (R_0/R_1)^6 + (R_0/R_2)^6} \quad (6)$$

where E is the total transfer efficiency, R_1 and R_2 are the distances to the first and second β-tyrosine sites, and F_1 and F_2 are the fractions of CF_1 molecules containing one and two NBD label(s). Given the kinetic analysis of NBD labeling in terms of eq 5, F_1 and F_2 can be calculated by using

$$F_1 = k_1(e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1) \quad (7)$$

$$F_2 = 1 + (k_1 e^{-k_2 t} - k_2 e^{-k_1 t})/(k_2 - k_1) \quad (8)$$

where

$$\text{mol of NBD/mol of CF}_1 = F_1 + 2F_2 \quad (9)$$

Plots of the fitted transfer efficiencies as a function of the NBD labeling stoichiometry are included in Figure 6 (solid lines). These fits gave separations of 44 and 39 Å, respectively, between the dark-site γ-sulfhydryl and the first and second β-tyrosine reactive sites and distances of 39 and 34 Å between the light-site γ-sulfhydryl and the same two β-tyrosine sites. The data were also fit to eq 6 with the assumption $R_1 = R_2$ (dashed lines). With this assumption, distances of 42 Å between the dark site and each of the β-tyrosines and 38 Å between the light site and the two β-tyrosines were obtained. The uncertainties in the quenching data do not permit an unequivocal distinction between the two models to be made.

DISCUSSION

In a series of previous studies, fluorescence resonance energy transfer measurements have been used to investigate the spatial relationships between the light-site γ-sulfhydryl, the γ-disulfide, and the nucleotide binding sites of the coupling factor complex from chloroplasts and the orientation of these sites relative to the membrane surface (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984). The combined results have been used to create a working model for the structural organization of the intact H^+-ATPase (Snyder & Hammes, 1984). In this model, the nucleotide binding sites from a slightly skewed triangle near the top of the extrinsic CF_1 portion of the enzyme, while the two sulfhydryl sites are located closer to the membrane, with the light site in the interior and the disulfide near the surface of the CF_1 complex. The light-site γ-sulfhydryl is symmetrically located with respect to the nucleotide binding sites. The γ-disulfide site, on the other hand, is located directly below nucleotide site 3, far from nucleotide sites 1 and 2.

The distance measurements presented in this paper permit the further inclusion of the dark-site γ-sulfhydryl within the above structural model. Construction of a three-dimensional model shows that all distances measured relative to the dark site are consistent with previously measured distances within ±10% and permit this sulfhydryl to be unambiguously located in the model. This is illustrated by the drawing in Figure 7. The three γ-sulfhydryl sites and the three nucleotide binding sites of CF_1 are shown as viewed parallel and perpendicular to the membrane surface. This model predicts a close association between the light- and dark-site γ-sulfhydryls, with an expected separation distance in the range 8–12 Å. Both of these sites are known to be located within rigid environments (Nalin et al., 1983; Cerione et al., 1983; Nalin & McCarty, 1984) and are approximately equidistant from the membrane surface in the reconstituted system (Cerione et al., 1983). Previous energy transfer measurements yielded a maximum separation of <27 Å between the two sites (Cerione et al., 1983); however, *o*-phenylenedimaleimide has been used to cross-link the light- and dark-site sulfhydryls, suggesting a separation of ≤10 Å (Weiss & McCarty, 1977). With such a short separation, conformational changes and the finite size

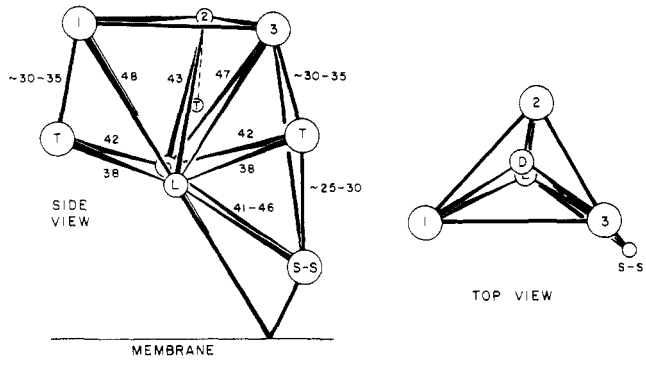

FIGURE 7: Schematic illustration of the spatial relationships between the nucleotide binding sites, the γ-sulfhydryls, the NBD-reactive β-tyrosines, and the membrane surface for the intact coupling factor complex suggested from fluorescence resonance energy transfer measurements. D and L are the dark- and light-site γ-sulfhydryls, respectively, S–S is the γ-disulfide, T represents the β-tyrosine sites, and the nucleotide binding sites are labeled 1–3. Views perpendicular and parallel to the bilayer surface are shown. The lines correspond to calculated distances, with the distances measured in this study given in angstroms. The relative locations of the β-tyrosine sites are approximates (see text for details). Not all features of the model are shown in the top view for clarity of presentation.

of the fluorescent probes may influence the distance measured directly by energy transfer.

Since the measured distance between the dark-site sulfhydryl and the γ-disulfide site is quite long (>40 Å), the inhibition of disulfide labeling caused by the presence of CPM at the dark site suggests the existence of long-range interactions between these two sulfhydryl sites. Before labeling by maleimide reagents can occur, the γ-disulfide must first be reduced through incubation with an excess of thiol reducing equivalents. Reduction of this disulfide, which activates ATPase activity in both the solubilized and intact coupling factor (Farron & Racker, 1970; Pick & Racker, 1979), has been postulated to give rise to a major conformational change within the γ subunit (Nalin & McCarty, 1984). The presence of a bulky ring system at the dark-site sulfhydryl may inhibit this conformational change and hence significantly alter the rate of reduction of the disulfide bond. Cross-linking of the CPM maleimide ring with neighboring amines (Wu et al., 1976) could also yield a similar effect. No inhibition of disulfide labeling was observed when the light site contained the CPM label (unpublished results).

The polypeptide chain stoichiometry of CF_1 suggested by recent molecular weight determinations (Moroney et al., 1983) implies the existence of three β-tyrosines within the enzyme complex that react with NBD-Cl. The modification of two β-tyrosines is readily observable, with the first β-tyrosine being significantly more reactive than the second. Reaction with a third β-tyrosine may occur, but apparently its reactivity cannot be distinguished from that of other groups on the enzyme that react with NBD-Cl. The mechanism for the reaction of NBD-Cl with the enzyme cannot be well-defined by the kinetic measurements. Consequently, the position of the NBD-tyrosines in the proposed structural model cannot be uniquely determined. However, some limitations can be placed on both the mechanism and the general placement of the NBD-tyrosines in the structure. A possible mechanism for the modification of the enzyme by NBD-Cl is that each of the tyrosines in CF_1 is uniquely reactive. If this were the case, previous energy transfer measurements (Cantley & Hammes, 1976; Cerione & Hammes, 1982), interpreted within the context of the model in Figure 7, would require that the unique tyrosines be close to one another and be located very near the dark- and light-site γ-sulfhydryls. The modest amount of energy transfer observed in this study indicates this is not the case. A second possibility is that two β-tyrosines of CF_1 react equally well with NBD-Cl but after the first tyrosine is modified on a single molecule, the second tyrosine on the same molecule reacts more slowly. This mechanism allows the NBD-tyrosines to be located within the proposed model and has been used to interpret the results reported here. A unique interpretation is hindered by the fact that modification of a third tyrosine cannot be clearly discerned. Therefore, the possibility that three tyrosines are equivalent before reaction with NBD occurs or that two are equivalent and one is not cannot be distinguished. This latter possibility might be due to the very tight binding of a single ADP to CF_1, although three nucleotide binding sites exist. In fact, the binding of nucleotides to the enzyme could alter the reactivity of the enzyme, further complicating matters. The kinetic analysis of the reaction of NBD-Cl with the enzyme and the accompanying energy transfer measurements have permitted calculation of a weighted average distance between the NBD-tyrosines and the light- and dark-site γ-sulfhydryls. For both sulfhydryl sites, the distances between the site and the first and second reacting tyrosines differ by about 5 Å. However, as shown under Results, an adequate fit of the data can be obtained by assuming the two NBD-tyrosines are equidistant from each sulfhydryl.

If the distances to the NBD-tyrosines reported in this and previous work are assumed to be qualitatively correct, the β-tyrosines can be placed approximately in the model of Figure 7. An important consideration in this model is that the diameter of CF_1 is approximately 100 Å (Howell & Moudrianakis, 1967). If the NBD-tyrosines are ~40 Å from the light- and dark-site sulfhydryls and are approximately symmetric with respect to the sulfhydryls, the tyrosines must be near the surface of CF_1. This location is consistent with the high reactivity of the tyrosines. In addition, the tyrosines must be quite far from each other, ~55–70 Å. If this placement of the β-tyrosines is to be consistent with previous energy transfer measurements (Cantley & Hammes, 1976; Cerione & Hammes, 1982), they must be located in a plane roughly parallel to and approximately 30–35 Å from the plane defined by the three nucleotide binding sites of CF_1. Each β-tyrosine is in a region directly below a corresponding nucleotide site. [This placement is a reinterpretation of previously obtained results (Cerione & Hammes, 1982). Previously, the first tyrosine labeled by NBD-Cl was assumed to be unique. If random labeling of two or three tyrosines is assumed and energy transfer between each nucleotide site and the nearest tyrosine is dominant, the calculated NBD–nucleotide separation for all three pairs is 30–35 Å.] The plane of the β-tyrosines is 5–10 Å above the dark- and light-site sulfhydryls and 25–30 Å above the γ-disulfide site. The tentative locations for the three β-tyrosines on the basis of this analysis are included in Figure 7. More complex interpretations of the data cannot be excluded, but are not required.

The use of fluorescence energy transfer techniques to obtain structural information about the organization of the γ-sulfhydryls, essential β-tyrosines, and nucleotide binding sites of CF_1 involves three basic assumptions. First, the site–site distance determinations contain the implicit assumption that the measured quenching of donor fluorescence is due solely to energy transfer to acceptor species and not to changes in environment induced by the binding of acceptor molecules. In support of this assumption, the two donor fluorophores used in this steady exhibit no changes in either their characteristic

polarizations or their corrected emission spectra upon binding of any combination of acceptor compounds. Second, modeling of the spatial relationships between the various sites relative to the dark-site sulfhydryl requires the additional assumption of structural equivalence between the intact and ϵ-deficient enzyme. This structural equivalence can be inferred from the following results: (1) the nucleotide binding properties of CF_1 are largely unaffected by removal of the ϵ subunit; (2) the fluorescence characteristics of maleimide probes covalently attached to the dark-site sulfhydryl are essentially independent of the presence or absence of the ϵ subunit on CF_1. Lastly, in the calculation of R_0 (eq 3), the isotropic limit for dipolar coupling between donor and acceptor has been assumed, namely, $\kappa^2 = {}^2/_3$. The steady-state polarizations of the various maleimide adducts at the three γ-sulfhydryl sites can be used to calculate a *maximum* uncertainty of $\pm 30\%$ in the determination of R_0 and hence the calculated distances due to uncertainties in κ^2 (Dale et al., 1979). However, the geometric constraints imposed by multiple distance determinations and the self-consistent nature of the spatial relationships obtained in this and other studies (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984; Nalin et al., 1985) suggest the actual errors in the calculated distances are no worse than $\sim 10\%$. Thus, the uncertainty in κ^2, even for fairly rigid environments, appears to be no more significant than the normal experimental uncertainties characteristic of energy transfer determinations. Several explanations are possible for this finding, for example, static isotropic averaging, multiple electronic transitions in the donor and acceptor, or fortuitous values of κ^2.

The structure in Figure 7 can be used to construct a plausible, although not unique, model for the organization of α-, β-, and γ-polypeptides in the coupling factor. In this model, the γ-polypeptide serves as a pedestal for the α- and β-polypeptides, with the α- and β-polypeptides alternating around the periphery of CF_1. This alternating arrangement is suggested by the extension of the β-polypeptide from the nucleotide sites to the NBD-tyrosines, by the location of an amine-containing residue of an α-polypeptide (Nalin et al., 1985), and by the fact that the nucleotide sites appear to be at the interface of α- and β-polypeptides (Bruist & Hammes, 1981; Kambouris & Hammes, 1985). The α- and β-polypeptides and the nucleotide sites clearly are asymmetric with respect to the γ-polypeptide. This asymmetry is very likely an important factor in the function of the enzyme. The location of the δ- and ϵ-polypeptides within CF_1 is not yet known, but energy transfer studies are currently under way to determine their placement in the model.

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