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Structural Map of the Dicyclohexylcarbodiimide Site of Chloroplast Coupling Factor Determined by Resonance Energy Transfer†

Bharati Mitra and Gordon G. Hammes*
Department of Chemistry, Cornell University, Ithaca, New York 14853
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ABSTRACT: Fluorescence resonance energy-transfer measurements were made on the membrane-bound chloroplast coupling factor. The distances from the *N,N'*-dicyclohexylcarbodiimide-binding site on the membrane-bound portion of the enzyme (CF_0) to the vesicle surface and to two sulfhydryl sites on the γ-polypeptide were determined. The dicyclohexylcarbodiimide-binding site was labeled with the fluorescent species *N*-cyclohexyl-*N'*-pyrenylcarbodiimide. The vesicle surface was labeled with *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine. Steady-state energy transfer between the fluorescent-labeled enzyme (energy donor) and varying concentrations of the ethanolamine derivative (energy acceptor) indicated that the distance of closest approach between the energy donor and the outer vesicle surface is 16–24 Å. Two specific sites on the γ-polypeptide were reacted with a coumarinylmaleimide derivative; one is a sulfhydryl that can be labeled only on the thylakoids under energized conditions (the "light" site), while the other is the disulfide site that regulates enzymatic activity. Energy-transfer measurements utilizing steady-state fluorescence and fluorescence lifetime methods indicated that the dicyclohexylcarbodiimide site is ~41 Å from the light site and ~50 Å from the γ-disulfide site. These distances are used to extend the current structural model of the chloroplast coupling factor.

The chloroplast ATP synthase catalyzes the photophosphorylation of ADP, coupling ATP synthesis to proton flow down a proton gradient. The enzyme complex consists of an extrinsic portion, chloroplast coupling factor 1 (CF_1),[1] and a membrane-imbedded pore (CF_0). CF_1 is easily solubilized and contains the substrate-binding sites. It is composed of five different polypeptide chains, α, β, γ, δ, and ε, with a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983). CF_0 contains the proton channel and has four different types of polypeptide chains (Pick & Racker, 1979), whose stoichiometry is not precisely known. The smallest of these, a protein

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CF_0, chloroplast coupling factor 0; DCCD, *N,N'*-dicyclohexylcarbodiimide; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl]maleimide; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid.

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of molecular weight 8000, contains a DCCD-binding site. DCCD is known to inhibit the proton pumping and, hence, ATP synthase activity of the enzyme. The DCCD-binding polypeptide is present in multiple copies of 6–12 per enzyme molecule (Foster & Fillingame, 1982).

Fluorescence resonance energy-transfer techniques have been used to elucidate the structure of CF_1 (Cerione & Hammes, 1982; Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Richter et al., 1985; McCarty & Hammes, 1987). However, the spatial relationships between sites on CF_1 and CF_0 have not been investigated with this technique. In the present study, a pyrene analogue of DCCD, NCP, was used to label CF_0. With NCP as a fluorescent energy donor, the distances between the DCCD-binding site and the membrane surface and between this site and two sites on the γ-polypeptide of CF_1 were measured.

Materials and Methods

Chemicals. Asolectin (crude soybean phospholipids), ATP (vanadium free), Triton X-100, ascorbate, phenazine methosulfate, and cholic acid, recrystallized prior to use (Kagawa & Racker, 1971), were from Sigma Chemical Co. Octyl glucoside was from Calbiochem. [^{32}P]P_i was from ICN. NCP, CPM, and NBD-PE were from Molecular Probes, Inc. DCCD and quinine sulfate were from Aldrich Chemical Co. All other chemicals were high-quality commercial grades. Solutions were prepared with deionized water.

Enzyme Preparation. The CF_1CF_0 complex was prepared from fresh market spinach by the procedure of Pick and Racker (1979), with modifications by Cerione and Hammes (1981). The partially purified enzyme was further purified on a 7–40% linear sucrose density gradient with 0.2% sodium cholate (Pick & Racker, 1979). The purified enzyme was stored at −70 °C, following quick-freezing in liquid nitrogen. When necessary, it was concentrated by using Sephadex G-25 medium (Cerione & Hammes, 1981). Protein concentrations were determined according to Bensadoun and Weinstein (1976), with bovine serum albumin as a standard. A molecular weight of 500 000 was assumed for CF_1CF_0.

To prepare enzyme specifically labeled on CF_0, thylakoids were incubated with NCP before solubilization of CF_1CF_0. Spinach leaves were homogenized in 200 mM K_2HPO_4 (pH 7.8), 350 mM NaCl, and 1 mM EDTA. The homogenate was pelleted and taken up in 0.4 M sucrose, 10 mM NaCl, and 50 mM Tricine (pH 7.8), at ~1 mg/mL chlorophyll. It then was incubated with NCP at ~0.5 μmol/mg of chlorophyll for 2–2.5 h at 4 °C. The NCP stock solution was 30–50 mM in dimethylformamide. After the reaction, unreacted NCP was removed by centrifugation of the protein and washing the precipitate with 10 mg/mL bovine serum albumin in 0.4 M sucrose, 10 mM NaCl, and 50 mM Tricine (pH 7.8) (Sigrist-Nelson & Azzi, 1979). After a second centrifugation and wash, the labeled CF_1CF_0 was extracted from the thylakoids and purified as usual.

CF_1CF_0, labeled with NCP on CF_0, was reacted with CPM to label the sulfhydryl of the γ-polypeptide that is exposed to reaction only under energized conditions (the γ-"light" site). First, the thylakoids were labeled with NCP, and excess probe was removed as described above. Next, all accessible sulfhydryl groups were blocked by incubating the thylakoids in 150 mM NaCl and 10 mM Tricine (pH 8.0) with 10 mM *N*-ethylmaleimide for 1 h. Unreacted *N*-ethylmaleimide was removed by centrifugation of the thylakoids and washing the precipitate with the same buffer. The thylakoids were taken up in 50 mM Na-Tricine (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, and 10 μM phenazine methosulfate, at 0.1–0.2 mg/mL chlorophyll, and incubated with 50 μM CPM for 5–10 min at room temperature. The reaction mixture was kept in a large crystallizing dish and illuminated from the top and bottom, with the light intensity at both surfaces being approximately 10^6 erg/(cm^2·s). The reaction was terminated by addition of dithiothreitol to 50 mM. CF_1CF_0 then was solubilized and purified as usual.

The disulfide on the γ-polypeptide also was labeled with CPM. The $(NH_4)_2SO_4$ precipitate containing crude, NCP-labeled CF_1CF_0 was dissolved in 20 mM Tricine (pH 8.0), 3 mM $MgCl_2$, and 200 mM sucrose (1–2 mg/mL enzyme). This enzyme preparation has variable amounts of the γ-disulfide reduced. In order to label specifically the γ-disulfide and to avoid labeling the exposed sulfhydryl of the γ-polypeptide ("dark" site), the enzyme was oxidized with $CuCl_2$ for 1 h (Mitra & Hammes, 1988), and 1 mM EDTA then was added to chelate the Cu^{2+}. This results in oxidation of the γ-disulfide. Accessible sulfhydryls then were blocked by reaction of the enzyme with 10 mM *N*-ethylmaleimide for 1 h in the same buffer. The reaction was quenched by addition of dithiothreitol to 50 mM. This mixture was incubated for 3 h to reduce the γ-disulfide. Excess dithiothreitol was removed by precipitating the enzyme twice with 45% $(NH_4)_2SO_4$ and taking up the precipitate in the above buffer. The CF_1CF_0 was incubated with 50 μM CPM for 5–10 min. Unreacted CPM was removed by addition of dithiothreitol to 10 mM, followed by precipitation of the enzyme with 45%-saturated $(NH_4)_2SO_4$. The precipitate was dissolved in the same buffer and precipitated a second time to ensure elimination of CPM. All of the above procedures were carried out at 4 °C. The doubly labeled enzyme was purified on a sucrose gradient in the usual way.

The polypeptide chain specificity of labeling by fluorescent probes was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the enzyme (Chua, 1980) and examination of the fluorescence of the protein bands when the gel was illuminated with ultraviolet light.

Reconstitution and Assays. CF_1CF_0 was reconstituted into asolectin vesicles by the following procedure (Krupinski & Hammes, 1986). Asolectin vesicles were prepared by sonicating crude asolectin in 150 mM KCl, 1 mM EDTA, and 10 mM Na-Tricine (pH 8.0), for 5 min under nitrogen. About 0.4 mg/mL purified enzyme was incubated with 7 mg/mL sodium cholate (pH 8.0), 5 mM Mg^{2+}, and 5 mg/mL vesicles for 10 min on ice, followed by passage of the enzyme through a Sephadex G-50 centrifuge column, preequilibriated with 150 mM KCl, 5 mM Mg^{2+}, 1 mM EDTA, and 10 mM Na-Tricine (pH 8.0).

Assays were carried out according to Carmeli and Racker (1973). Typical [^{32}P]P_i–ATP exchange activities of reconstituted CF_1CF_0 were 100–150 nmol of P_i min^{-1} (mg of enzyme)$^{-1}$ at 37 °C. Phenazine methosulfate dependent ATP synthase activity of the thylakoids was assayed at 30 °C, using two Kodak 600H slide projectors equipped with ELH lamps (300 W) as the light source. The [γ-^{32}P]ATP synthesized was measured by molybdate extraction of the reaction mixture. Typical activities of thylakoids were 20–30 μmol (mg of chlorophyll)$^{-1}$ min^{-1}.

Energy-Transfer Measurements. The energy-transfer measurements between the NCP-labeled CF_0 of CF_1CF_0 (energy donor) and the vesicle surface were performed as follows. Vesicles containing NBD-PE (energy acceptors) were prepared by addition of 0.04–0.8 mM NBD-PE in dimethyl sulfoxide to 20 mg/mL asolectin in 150 mM KCl, 10 mM Na-Tricine (pH 8.0), and 1 mM EDTA. The vesicles were

sonicated under nitrogen for 5 min. Gradient-purified NCP-labeled CF_1CF_0 was reconstituted in these vesicles as described previously. NCP-labeled enzyme reconstituted in asolectin vesicles containing 10% dimethyl sulfoxide served as a control (energy donor in the absence of energy acceptor). The reconstituted samples were used directly for energy-transfer measurements.

Energy-transfer measurements between the NCP-labeled CF_0 and the CPM-labeled γ-polypeptide were performed by reconstitution of NCP-labeled CF_1CF_0 into asolectin vesicles (energy donors in the absence of acceptor) and NCP–CPM-labeled CF_1CF_0 into vesicles (energy donor in the presence of energy acceptor). In all cases, unlabeled CF_1CF_0 reconstituted into asolectin vesicles served as a control to correct for light scattering of the samples.

Concentrations of probes and labeling stoichiometries were determined by use of extinction coefficients of 3.02×10^4 M^{-1} cm^{-1} at 387 nm for CPM–protein (Sippel, 1981) and 2.24×10^4 M^{-1} cm^{-1} at 465 nm for NBD-PE in sonicated vesicles (Cerione et al., 1983). The extinction coefficient of the acetic acid derivative of NCP in ethanol was determined to be 4.33×10^4 M^{-1} cm^{-1}. This extinction coefficient was used to determine the amount of NCP reacted with CF_1CF_0 as follows. The NCP-labeled polypeptide from sucrose density gradient purified CF_1CF_0 was extracted in 1-butanol by the procedure of Sigrist et al. (1977). The 1-butanol extract was concentrated at 0 °C under vacuum and acidified with 8 mM HCl. Its absorption spectrum then was compared with that of the acetic acid derivative of NCP in acidic ethanol. Labeling stoichiometries were calculated after corrections for probe–probe spectral overlap and light scattering due to enzyme and vesicles.

Spectroscopic Measurements. Absorbance measurements were made with 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. Steady-state polarization measurements were corrected for unequal transmission of horizontally and vertically polarized light (Cerione & Hammes, 1982). The quantum yield for NCP-labeled CF_1CF_0 was measured by a comparative method (Parker & Rees, 1960). The fluorescence standard used was quinine sulfate in 0.1 N H_2SO_4, which was assumed to have a quantum yield of 0.70 (Scott et al., 1970).

Measurements of steady-state fluorescence resonance energy transfer were corrected for light scattering, inner filter effects, and acceptor stoichiometry. Corrections for light scattering were made by subtraction of the apparent fluorescence of unlabeled CF_1CF_0 reconstituted into asolectin vesicles. The contribution of inner filter effects to the steady-state quenching ratios was calculated directly from the measured acceptor absorbance at the donor excitation and emission wavelengths, respectively; the inner filter effects were typically <5%. Finally, when necessary, the fluorescence was corrected for incomplete labeling of acceptor sites by dividing the fluorescence by the fraction of sites labeled.

Fluorescence lifetime measurements were made with an Edinburgh Instruments nanosecond fluorescence spectrometer. The excitation light, provided by a spark gap flash lamp, was passed through a monochromator. Decay spectra were collected with a 380-nm emission interference filter (10-nm band-pass; Ditric Corp.). Background and light-scattering corrections, made by measuring the apparent emission of unlabeled reconstituted CF_1CF_0, were subtracted from the sample fluorescence decay. Lamp–pulse profiles were obtained from the light scattering of a 0.1% Ludox solution with the emission filter removed. The data were analyzed by a nonlinear least-squares routine provided by Edinburgh Instruments.

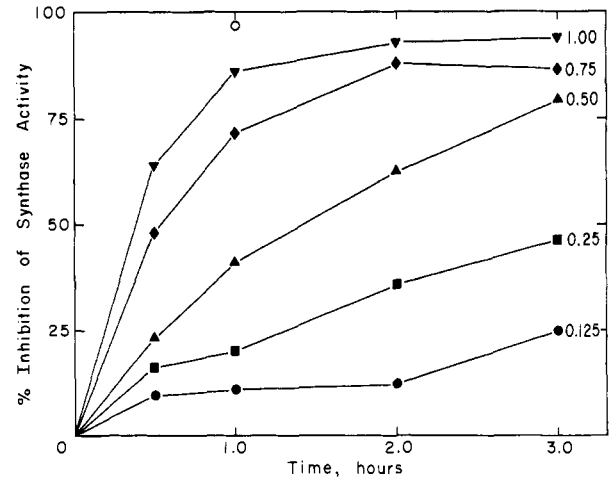

FIGURE 1: Plot of the percent inhibition of ATP synthase activity vs time of incubation with NCP in 0.4 M sucrose, 10 mM NaCl, and 50 mM Tricine (pH 7.8), at 0 °C. The numbers denote the NCP concentrations in micromoles per milligram of chlorophyll in the incubation mixture. The point (O) indicates incubation with DCCD at 0.25 μmol/mg of chlorophyll under identical conditions.

RESULTS

Reaction of CF_1CF_0 with NCP inhibits the ATP synthase activity of thylakoids. Figure 1 shows the time dependence of inhibition at different concentrations of NCP in the incubation mixture. NCP was found to be less reactive than DCCD (open circle in Figure 1); however, complete inhibition was obtained at high concentrations of NCP over sufficiently long incubation times. The rate of synthase activity of thylakoids before reaction with NCP was ~27 μmol (mg of chlorophyll)$^{-1}$ min^{-1} in this experiment.

The unreacted NCP is nonfluorescent in aqueous and nonaqueous solutions. The acetic acid derivative of NCP, prepared by reacting 50 μM NCP with 30 mM acetic acid in ethanol for 1–3 h, displayed a highly structured fluorescence spectrum with emission maxima at 375, 387, and 396 nm. However, in aqueous solutions, the acetic acid derivative of NCP showed a broad, structureless spectrum (data not shown).

In order to label CF_0 specifically with NCP, and to facilitate removal of unreacted NCP, CF_1CF_0 was labeled with NCP before its solubilization from the membrane. Unreacted NCP was then effectively removed by bovine serum albumin washes and also by the purification steps in the isolation of the enzyme. The solubilized, labeled enzyme displayed a broad, structureless, fluorescence emission spectrum in 150 mM KCl, 10 mM Na-Tricine (pH 8.0), and 1 mM EDTA. However, when the purified, labeled enzyme was reconstituted into asolectin vesicles, a fluoresence emission spectrum was obtained which was very similar to the acetic acid derivative of NCP in ethanol (Figure 2). This indicates that the environment around bound NCP is quite hydrophobic, "ethanol-like" rather than "water-like". The steady-state fluorescence properties of NCP bound to CF_0, namely, the fluorescence emission maxima, the quantum yield, and the polarization, are given in Table I. The labeling was highly specific as checked by the fluorescence of sodium dodecyl sulfate–polyacrylamide gels; the violet fluorescence due to reacted NCP was confined to the c-polypeptide of CF_0. Stoichiometries of bound NCP ranged from 0.7 to 1.0 mol/mol of CF_1CF_0.

Table I: Steady-State Energy-Transfer Parameters for NCP Bound to CF_0[a]

fluorescence max (nm)	Q_D	P	acceptor	location	absorbance max (nm)	R_0 (Å)	R (Å)
375, 387, 396	0.2	0.2	NBD-PE	membrane	465	27.3	16
			CPM	DiSH	465	33.5	50
			CPM	L	465	33.5	40

[a] Abbreviations: DiSH, γ-disulfide site; L, the γ-sulfhydryl labeled under energized conditions; P, steady-state polarization measured at fluorescence excitation and emission maxima; R, distance between donor and acceptor sites calculated from steady-state measurements. For NBD-PE in the membrane, R is the distance of closest approach between donor and acceptors.

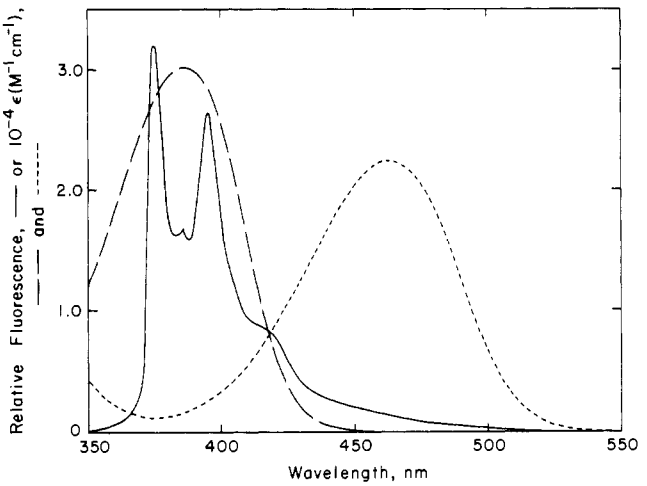

FIGURE 2: Spectral overlap of the fluorescence emission for NCP (—) bound to CF_0, with the extinction coefficient of NBD-PE on the vesicle surface (---) and CPM attached to the γ-light and disulfide sites (– –). The fluorescence excitation was at 343 nm. All spectra were taken at 4 °C in 5 mM Mg^{2+}, 150 mM KCl, 10 mM Tricine (pH 8.0), and 1 mM EDTA.

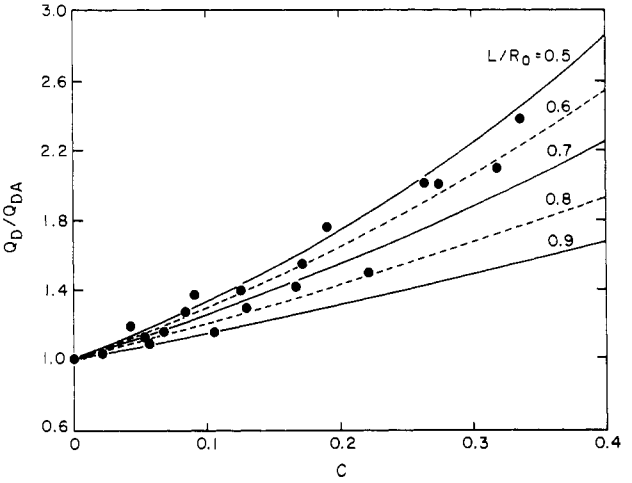

FIGURE 3: Plot of the ratio of donor quantum yield in the absence and presence of acceptor (NBD-PE), Q_D/Q_{DA}, vs the normalized acceptor concentrations, $C = \sigma R_0^2$, for reconstituted NCP-labeled CF_1CF_0. The fluorescence measurements were made in 5 mM Mg^{2+}, 150 mM KCl, 10 mM Tricine (pH 8.0), and 1 mM EDTA. Fluorescence excitation was at 343 nm, and emission was measured at 375 nm. The curves represent the theoretical quenching expected for the given values of L/R_0 calculated through Monte Carlo simulations.

CF_1CF_0 labeled on the thylakoids was detergent-solubilized and purified on a linear sucrose density gradient. The gradient profiles for labeled and unlabeled CF_1CF_0 were very similar to those obtained by Cerione et al. (1983). Typical ATP–P_i exchange activities of crude unlabeled CF_1CF_0 were 100–150 nmol/(mg·min). The ATP–P_i exchange activity of NCP-labeled CF_1CF_0 was inhibited 75–100% relative to that of the unlabeled enzyme for a stoichiometry of 0.7–1.0 mol of NCP/mol of CF_1CF_0.

The distance between the NCP-binding site on reconstituted CF_1CF_0 and the vesicle surface was measured by performing energy-transfer measurements with NCP as the energy donor and NBD-PE distributed on the vesicle surface as the energy acceptor. The absorption spectrum of NBD-PE in vesicles is included in Figure 2. Since the NCP-binding site is presumably inside the membrane, the results were analyzed in terms of energy transfer between a single donor on one plane and a uniform distribution of acceptors in another plane, the outer vesicle surface. The maximum contribution to the energy transfer due to a second plane of acceptors, the inner vesicle surface, then was calculated by assuming a bilayer width of 37 Å (Huang & Mason, 1978) and used as a correction factor for determination of the distance of NCP from the outer surface.

For the case of donors and acceptors distributed on two planes, the ratio of the quantum yields in the presence and absence of acceptors, Q_{DA}/Q_D, is given by (Shaklai et al., 1977)

$$Q_{DA}/Q_D = (1/\tau_D)\int_0^\infty \exp[-t/\tau_D - \sigma S(t)]dt$$

$$S(t) = \int_L^\infty \{1 - \exp[(-t/\tau_D)(R_0/R)^6]\}2\pi R dR \quad (1)$$

where τ_D is the fluorescence lifetime of the donor, σ is the surface density of energy acceptors, t is the time, R is the distance between a donor and an acceptor, and L is the distance of closest approach between the donors and acceptors. R_0 is given by

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

Here κ^2 is the orientation factor for dipolar coupling between donors and acceptors, J is the spectral overlap integral, and n is the refractive index of the medium. The value of κ^2 was assumed to be $^2/_3$, the dynamic average.

Equation 1 can be solved numerically and by a series approximation (Dewey & Hammes, 1980). Wolber and Hudson (1979) have presented an empirical representation of the numerical integration for some cases. Theoretical quenching profiles also can be obtained through a method of Monte Carlo simulations (Snyder & Freire, 1982). The distance of closest approach, L, can be calculated by fitting the experimental data to these theoretical curves.

The ratio Q_D/Q_{DA} for NCP-labeled CF_1CF_0 reconstituted into NBD-PE-labeled vesicles is shown in Figure 3 as a function of c ($=\sigma R_0^2$). The density of acceptor molecules, σ, was calculated by assuming a uniform distribution on the outer and inner vesicle surfaces and by using a molecular weight of 855 for NBD-PE and 740 for asolectin. R_0 (Table I) was calculated with eq 2, and J was calculated from the spectra in Figure 2. The curves in Figure 3 represent Monte Carlo solutions for the given values of L/R_0, with the assumption of a single donor and an infinite plane of acceptors. Minimization of the root mean square deviation of the experimental data from the curves gave $L/R_0 = 0.59$, or $L = 16$ Å. This treatment assumes that the donor fluorescence is quenched only by the acceptors on the outer vesicle surface. The

Table II: Fluorescence Lifetime Energy-Transfer Parameters for NCP Bound to CF_0[a]

enzyme sample	A_1	τ_1 (ns)	A_2	τ_2 (ns)	τ_{av} (ns)	R (Å)
NCP–CF_1CF_0	0.63 ± 0.01	13.1 ± 0.9	0.37 ± 0.01	89.2 ± 2.6	41.3	
NCP–CPM–CF_1CF_0 (DiSH)	0.69 ± 0.01	12.0 ± 0.7	0.31 ± 0.01	86.9 ± 2.4	35.2	50
NCP–CF_1CF_0	0.58 ± 0.01	15.0 ± 0.5	0.42 ± 0.01	74.9 ± 0.9	40.2	
NCP–CPM–CF_1CF_0 (L)	0.65 ± 0.01	12.3 ± 0.5	0.35 ± 0.01	65.5 ± 0.7	30.9	41

[a] Fluorescence lifetimes, τ_i, and amplitudes, A_i, with $A_1 + A_2 = 1$, as defined in eq 5 and 6. R is the distance between donor and acceptor sites calculated from energy transfer due to changes in the average fluorescence lifetimes. L designates the site labeled under energized conditions and DiSH the disulfide on the γ-polypeptide.

maximum error in the value of L introduced by this assumption can be estimated as follows. The error will be a maximum if it is assumed that the donor is separated by a distance of 21 Å from the inner surface, based on a bilayer width of 37 Å. The theoretical curve for Q_D/Q_{DA} vs C with $L/R_0 = 0.8$ (21/27) should then describe the maximum quenching of the donor by acceptors on the inner vesicle surface only. Subtraction of this curve from experimental data and fitting the resulting data to the theoretical curves give $L/R_0 = 0.9$ or $L = 24$ Å. This is the maximum possible value of L, since the distance of closest approach is not necessarily the perpendicular distance from the plane, and, therefore, the distance of the donor from the inner plane can be greater than 21 Å.

The efficiency of energy transfer between the NCP-labeled CF_0 and the CPM-labeled γ-polypeptide was obtained from the relationship:

$$E = 1 - Q_{DA}/Q_D = 1 - \tau_{DA}/\tau_D \quad (3)$$

where Q_{DA}/Q_D and τ_{DA}/τ_D are the ratios of the quantum yields and fluorescence lifetimes, respectively, in the presence and absence of energy acceptors. The distance R between the donor and acceptor sites was calculated with (Förster, 1959)

$$E = (R_0/R_i)^6/[1 + (R_0/R_i)^6] \quad (4)$$

The energy-transfer efficiency for NCP labeled on CF_0 and CPM labeled at two different sites on the γ-polypeptide was calculated by measurement of both the ratio of the steady-state quantum yields and the ratio of the fluorescence lifetimes. The steady-state quantum yields were corrected for inner filter effects and protein concentrations. The background signal due to light scattering from vesicles and asolectin impurity fluorescence was determined by measurement of the fluorescence of unlabeled CF_1CF_0 reconstituted in vesicles, which was subtracted from the observed fluorescence in each case. The results of the steady-state energy-transfer measurements are summarized in Table I.

For fluorescence lifetime measurements, the fluorescence decay data were fit to the equation:

$$F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \quad (5)$$

where τ_1 and τ_2 are the fluorescence lifetimes and A_1 and A_2 are constants which were normalized to $A_1 + A_2 = 1$. An average lifetime, representing the normalized area under the decay curve, was calculated from

$$\tau_{av} = A_1\tau_1 + A_2\tau_2 \quad (6)$$

The energy-transfer efficiency was calculated from the ratio of τ_{av} for NCP-labeled CF_1CF_0 and NCP–CPM-labeled CF_1CF_0 (eq 3). The background signal due to scattering was corrected for by subtraction of the counts due to unlabeled enzyme from the counts due to the labeled samples. The light scattering from asolectin vesicles was quite large. As a result, even after subtraction of the background counts, small variations in vesicle concentration from sample to sample produced very large counts in the first few channels due to scattered light. In order to ensure a good fit of the data to eq 5, the first ~5 ns after the peak counts were excluded from the fit

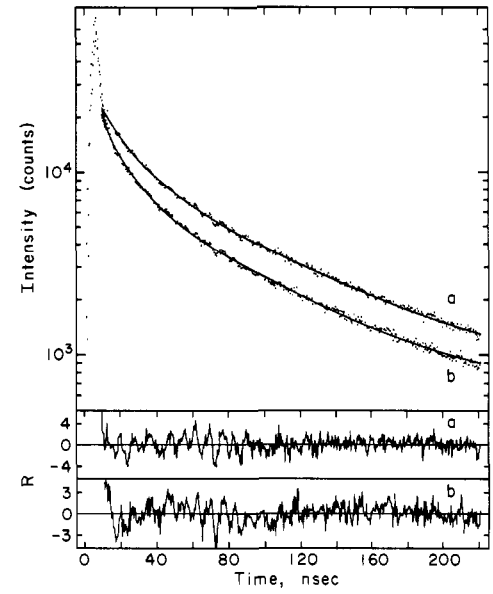

FIGURE 4: Plot of the fluorescence emission decay of NCP-labeled CF_1CF_0, in the absence (a) and presence (b) of CPM labeled at the γ-light site, vs time. The enzyme was in 5 mM Mg^{2+}, 150 mM KCl, 10 mM Tricine (pH 8.0), and 1 mM EDTA. The solid lines were calculated with eq 5, with the best-fit parameters in Table II. The normalized residuals, R, are shown at the bottom. (R is an approximate statistical measure of the normalized deviation from the data. $R_i \approx [Y(i)]^{-1/2}\,[Y(i) - F(i)]$, where $Y(i)$ is the fluorescence decay data and $F(i)$ is the value of the fitted function at time i.)

for all the samples. Typical decay curves for the acceptor on the "light site" and the disulfide are shown in Figures 4 and 5, respectively, together with the best-fit curves to eq 5. The best-fit parameters are summarized in Table II.

The sulfhydryl on the γ-polypeptide that is exposed to reaction only under energized condition was labeled with the energy acceptor CPM. In order to obtain a high stoichiometry of ~1.0 mol/mol of CF_1CF_0, the incubation of the enzyme with CPM was carried out for 10 min. This resulted in a small amount of labeling of the α- and β-polypeptides in addition to the γ-polypeptide, as visualized on sodium dodecyl sulfate–polyacrylamide gels; however, there was no fluorescent label on the ε-polypeptide. The steady-state energy-transfer efficiency was 0.26, which corresponds to a distance of 40 Å from the NCP site to the γ-"light" site. The efficiency due to changes in the average lifetime was 0.23, corresponding to a distance of 41 Å (Table I).

The γ-disulfide also was labeled with CPM at a stoichiometry of ~2.0 mol/mol of CF_1CF_0. Again the high stoichiometry resulted in a small amount of the α- and β-polypeptides being labeled. The efficiencies due to steady-state energy transfer and to changes in average lifetimes were identical, 0.15, yielding a separation distance of 50 Å between the NCP site and the γ-disulfide site. This assumes that the two sulfhydryls remain in close proximity after reduction (Snyder & Hammes, 1984), so that the effective extinction coefficient of the acceptor is twice that of a single acceptor

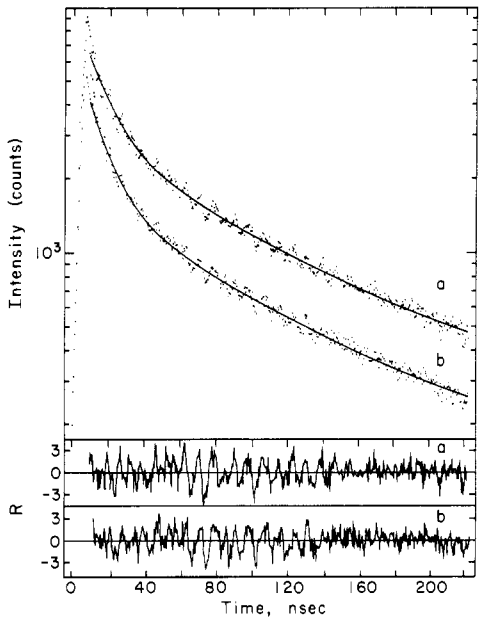

FIGURE 5: Plot of the fluorescence emission decay of NCP-labeled CF_1CF_0, in the absence (a) and presence (b) of CPM labeled at the γ-disulfide site, vs time. The enzyme was in 5 mM Mg^{2+}, 150 mM KCl, 10 mM Tricine (pH 8.0), and 1 mM EDTA. The solid lines were calculated with eq 5, with the best-fit parameters in Table II. The normalized residuals, R, are shown at the bottom, where R is defined in the legend to Figure 4.

molecule. The net result is that R_0 is increased by a factor of $2^{1/6}$.

Discussion

In this study, fluorescence resonance energy-transfer techniques have been used to measure the distances from the DCCD-binding site on the c-polypeptide of CF_0 to the membrane surface and to the γ-polypeptide of CF_1. Energy-transfer techniques have been previously used to construct a spatial map of CF_1 (Snyder & Hammes, 1984; Richter et al., 1985; McCarty & Hammes, 1987); however, information about the location of specific sites on CF_0 has been quite limited. Using a spin-labeled analogue of DCCD, Sigrist-Nelson and Azzi (1979) showed that the maximal distance between the multiple copies of the DCCD-binding protein in CF_0 is 15–20 Å. NCP is an analogue of DCCD in which one of the cyclohexyl rings is replaced by pyrene. As shown in Figure 1, NCP is an effective inhibitor of CF_1CF_0 although its rate of reaction with the enzyme is slower than that of DCCD. For the mitochondrial H^+-ATPase, Pringle and Taber (1985) showed that NCP and DCCD compete for the same binding site. Because NCP labels a specific site and forms a fluorescent product after reaction with CF_1CF_0, it can be used to probe the spatial location of the DCCD-binding site with respect to the membrane surface and specific sites on the reconstituted chloroplast coupling factor.

In the energy-transfer experiments, NCP serves as the donor, while NBD-PE in the vesicles and CPM attached to the γ-polypeptide serve as the acceptors. For the NCP labeling conditions used, a [NCP]/[CF_1CF_0] stoichiometry of 0.7–1.0 mol/mol was obtained. There are 6–12 copies of the c-polypeptide per CF_1CF_0 complex; however, their mode of arrangement is not known. In this work, it is assumed that for a NCP-binding stoichiometry of 0.7–1.0 mol/mol of CF_1CF_0, the same specific site on a single c-polypeptide is labeled in each enzyme molecule. This is consistent with the complete loss in exchange activity when one site per molecule is modified, although a random labeling cannot be strictly excluded.

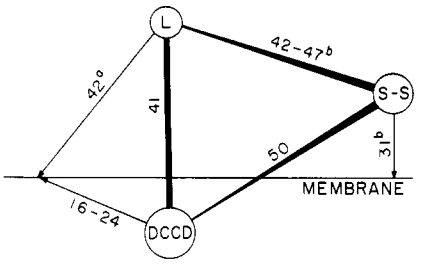

FIGURE 6: Model of the spatial relationships between the DCCD-binding site and the membrane surface, the γ-"light" site, and the γ-disulfide sites suggested by the energy-transfer measurements. The distances are in angstroms. For completeness, the distance between the γ-"light" and disulfide sites and the distances of these sites from the membrane surface are included. [(a) From Cerione et al. (1983); (b) from Snyder and Hammes (1984).]

The high quantum yield and polarization suggest that this site is quite hydrophobic and rigid, although some rotational freedom exists since the polarization is far from the maximum possible value. This is supported by the observation that the very hydrophobic probe NCP does not react with the solubilized enzyme but reacts with the enzyme in intact thylakoids or in reconstituted vesicles. The reaction is extremely specific: even when the thylakoids were incubated with NCP at 2.5 μmol/mg of chlorophyll for 3–4 h, the label was confined to the c-polypeptide as visualized on a sodium dodecyl sulfate–polyacrylamide gel. The difference in the emission spectra of the solubilized and reconstituted enzyme is quite marked; this served as a means of checking whether the enzyme was properly reconstituted.

The NCP-labeled enzyme can be characterized by two fluorescence lifetimes of ~15 and ~80 ns, with the biexponential decay curve being dominated by the shorter lifetime. The exclusion of the first ~5 ns from the data fit may cause some uncertainty in the shorter lifetime. However, this does not significantly affect the energy-transfer results since the shorter lifetime contributes a relatively small amount to the average lifetime.

In measuring the distance from the NCP site to the membrane surface, it was assumed initially that energy transfer occurs only to the acceptors on the outer surface. This gives a distance of closest approach of 16 Å. If the contribution to the energy transfer from the inner surface of acceptors is considered, then the maximum distance to the outer surface becomes 24 Å. Since the distances involved here are closest approach distances, which are not necessarily perpendicular to the surfaces, the distance of closest approach of the DCCD site from the outer vesicle surface is between 16 and 24 Å. For the mitochondrial H^+-ATPase, the DCCD site is 14–18 Å from the membrane surface (Pringle & Taber, 1985).

The NCP fluorescence emission spectrum has a good overlap with the CPM absorbance spectrum (Figure 2), resulting in a value for R_0 of 33.5 Å. The energy-transfer efficiencies obtained from steady-state and fluorescence lifetime measurements are in good agreement. An average distance of ~50 Å from the γ-disulfide site and ~41 Å from the γ-light site was obtained. The assumptions and uncertainties in distances measured by fluorescence resonance energy transfer have been discussed elsewhere (Snyder & Hammes, 1985); the error in the distances is ~10%.

The distances obtained in this study, together with some obtained previously, allow construction of the working model for CF_1CF_0 presented in Figure 6. In this model, the DCCD site is placed directly below the γ-"light" site, since their distance of separation, ~41 Å, is much smaller than the sum of their closest distances to the membrane surface (not nec-

essarily the perpendicular distances). The γ-disulfide site, on the other hand, is located above and to one side of the DCCD site. The distance between these two sites, ~50 Å, is greater than the sum of their distances to the membrane surface. Therefore, the DCCD site is definitely not directly below the γ-disulfide site. In other words, the DCCD site and the disulfide cannot be on the same side of the molecule. This model can be used as the basis for future experiments.

Registry No. ATPase, 9000-83-3; DCCD, 538-75-0.

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