Membrane-Protein Structural Mapping of Chloroplast Coupling Factor in Asolectin Vesicles†

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ABSTRACT: The spatial relationship of specific sites on chloroplast coupling factor, reconstituted in asolectin vesicles, to the bilayer surface has been studied with fluorescence methods. Fluorescence resonance energy transfer measurements have been used to map the distances of closest approach of the *N,N'*-dicyclohexylcarbodiimide-binding site and the disulfide on the γ-polypeptide to the bilayer center. The dicyclohexylcarbodiimide site was labeled with *N*-cyclohexyl-*N'*-pyrenylcarbodiimide and the γ-disulfide site with a coumarinyl derivative. The bilayer center was labeled with 25-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-*N*-methylamino]-27-norcholesterol. The distances obtained, 15 and 43 Å, respectively, were combined with previous measurements of the distance of closest approach between these sites and the membrane surface to estimate the perpendicular distances of the sites from the membrane surface. The depth of the dicyclohexylcarbodiimide site was also determined by studying the quenching of fluorescence by 5-, 7-, 12-, and 16-doxylstearic acids. The model developed suggests that the dicyclohexylcarbodiimide site is 6–10 Å below the membrane surface and the γ-disulfide is 16 Å above the membrane surface. The distances measured are subject to a considerable uncertainty, but the proposed model provides a useful starting point for further structural studies.

The spinach chloroplast ATP synthase catalyzes the synthesis of ATP from ADP and P_i, coupling ATP synthesis to dissipation of a proton gradient across the thylakoid membrane. The protein is a multisubunit complex, consisting of a membrane-imbedded pore, CF_0,[1] and an extrinsic portion, CF_1. CF_1 can be easily solubilized from the membrane. It has five different polypeptide chains, α, β, γ, δ, and ε, with a probable stoichiometry of $\alpha_3\delta_3\gamma\beta\epsilon$ (Moroney et al., 1983). CF_0 contains four different types of polypeptide chains (Pick & Racker, 1979), whose stoichiometry is not yet established. The smallest of the CF_0 polypeptides is a hydrophobic protein of molecular weight 8000, which contains a DCCD-reactive site. DCCD is a potent inhibitor of the proton pumping activity. The DCCD-binding protein is present in multiple copies of 6–12 per CF_1CF_0 complex (Foster & Fillingame, 1982).

Detailed structural information about CF_1 and the location of the DCCD-binding site have been obtained by mapping distances between specific sites on the protein and the membrane by fluorescence resonance energy transfer techniques (Cerione et al., 1983; Snyder & Hammes, 1984, 1985; Richter et al., 1985; McCarty & Hammes, 1987; Mitra & Hammes, 1989). However, resonance energy transfer techniques cannot be used to obtain the perpendicular distance from a point in the protein to the plane of the membrane. In this work, a combination of energy transfer techniques and fluorescence quenching by spin-labels was used to obtain the perpendicular distance between two specific sites on CF_1CF_0 and the membrane surface. These, together with previously mapped distances, are used to extend the existing structural model of CF_1CF_0.

Materials and Methods

Chemicals. Asolectin (crude soybean phospholipids), ATP (vanadium free), Triton X-100, and cholic acid, recrystallized prior to use (Kagawa & Racker, 1971), were from Sigma Chemical Co. Octyl glucoside was from Calbiochem. NCP, CPM, NBD-PE, NBD-MANC, and 5-, 7-, 12-, and 16-doxylstearic acids were from Molecular Probes, Inc. Cholestane spin-label was 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 commercial spinach by the procedure of Pick and Racker (1979), with modifications by Cerione and Hammes (1981). To ensure removal of the major contaminant, ribulose-1,5-bisphosphate carboxylase, the thylakoids were washed twice with unbuffered 10 mM NaCl. The enzyme was purified on a 7–40% linear sucrose gradient with 0.2% sodium cholate and stored at −70 °C, following quick freezing in liquid nitrogen.

CF_1CF_0, specifically labeled with NCP at the DCCD-reactive site of the c polypeptide (Mitra & Hammes, 1989) or with CPM on the disulfide site of the γ-polypeptide (Snyder & Hammes, 1984), was prepared according to known procedures. The labeling of the disulfide was performed on the partially purified enzyme, which subsequently was purified on a sucrose density gradient. The enzyme is fully active when the γ-disulfide is labeled but inactive when the DCCD site is labeled (Mitra & Hammes, 1989; Snyder & Hammes, 1984).

The specificity of labeling by the two fluorescent probes was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the enzyme (Chua, 1980). Examination of the fluorescence of the protein bands during illumination with ultraviolet light showed that the NCP fluorescence was confined to the c polypeptide of CF_0 and >95% of the CPM

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CF_0, chloroplast coupling factor O; DCCD, *N,N'*-dicyclohexylcarbodiimide; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl]maleimide; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; NBD-MANC, 25-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-*N*-methylamino]-27-norcholesterol; 5-, 7-, 12-, and 16-SA, 5-, 7-, 12-, and 16-doxylstearic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid.

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fluorescence was specific to the γ-polypeptide.

Protein concentrations were determined according to Bensadoun and Weinstein (1976), with bovine serum albumin as the standard. CF_1CF_o was assumed to have a molecular weight of 500000

CF_1CF_o was reconstituted into asolectin vesicles by the octyl glucoside dilution procedure (Takabe & Hammes, 1981). Vesicles were prepared by sonicating a suspension of asolectin in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA. Purified CF_1CF_o was reconstituted into these vesicles by addition of 0.7% octyl glucoside to a 1:1 mixture of enzyme (0.5–1 mg/mL) and vesicles (20 mg/mL). After incubation at 0 °C for ~5 min, a 30-μL aliquot was diluted to 2.5 mL with 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA.

Fluorescence Quenching Experiments. Experiments involving the fluorescence quenching of NCP-labeled CF_1CF_o by spin-labeled stearic acids were performed in two ways. In the first, the vesicle samples were prepared by dissolving asolectin and the doxylstearic acids in chloroform. The solvent then was completely evaporated under vacuum. The residue was vortexed with 20 mM Na-Tricine (pH 8.0), 1 mM EDTA, and the required concentration of stearic acid to give an asolectin concentration of 20 mg/mL. Finally, the lipids were sonicated for ~5 min to give unilamellar vesicles. The NCP-labeled enzyme was reconstituted in these vesicles as described previously. The vesicles were also prepared by addition of aliquots of doxylstearic acid (10 mM in methanol) directly to the reconstituted NCP-labeled CF_1CF_o. Measurements were made after 5 min of equilibration. The decrease in the fluorescence intensity at 375 nm (excitation 343 nm) was recorded as a function of increasing concentration of doxylstearic acids in the vesicles. Unlabeled enzyme reconstituted into vesicles served as a control.

To measure the depth of the NBD group in NBD-MANC- or NBD-PE-labeled asolectin vesicles, samples were prepared by mixing the required amounts of asolectin, NBD-labeled lipid, and a specific doxylstearic acid in chloroform. After the solvent had been evaporated, unilamellar vesicles were prepared as described above. The molar ratio of NBD-labeled lipid to asolectin was 1:100. Quenching experiments were also performed by directly adding aliquots of the spin-labels to the NBD-labeled vesicles. Fluorescence was recorded at 530 nm (excitation 465 nm) for NBD-labeled vesicles. The fluorescence intensity was corrected for light scattering in all experiments.

The concentrations of spin-labeled stearic acids were determined by double integration of the first-derivative ESR spectra of ~0.5 mM solutions in methanol. A standard solution of cholestane spin-label was used for comparison.

Energy Transfer Measurements. Energy transfer measurements were performed with the reconstituted enzyme. NCP at the DCCD site or CPM at the γ-disulfide site served as the two energy donors. The energy acceptor was NBD-MANC, distributed in the interior of the vesicle bilayer. NBD-MANC-labeled vesicles were prepared by addition of NBD-MANC in dimethylformamide to the asolectin suspension followed by sonication under nitrogen for 5 min. Typically, NBD-MANC:asolectin molar ratios were 0–1.5:100. The dimethylformamide in the sonication mixture was 1% (v/v). NCP- or CPM-labeled enzyme reconstituted in asolectin vesicles containing 1% dimethylformamide served as a control (energy donor in the absence of energy acceptor).

Fluorescence was recorded with an excitation wavelength of 343 nm and an emission wavelength of 375 nm for the NCP-labeled protein and with an excitation wavelength of 387 nm and an emission wavelength of 465 nm for the CPM-labeled protein. Samples were kept at 0 °C. The ratio of quantum yields of the donor in the absence or presence of acceptors was recorded as a function of increasing concentration of NBD-MANC in the vesicles. The fluorescence intensity was corrected for light scattering and inner-filter effects. Corrections for light scattering were made by subtraction of the signal due to unlabeled protein reconstituted into vesicles. Inner-filter effects were calculated from the measured acceptor absorbance at the donor excitation and emission wavelengths.

Probe concentrations and labeling stoichiometries were determined by use of extinction coefficients of 3.02×10^4 M^{-1} cm^{-1} at 387 nm for CPM-labeled protein (Sippel, 1981), 4.33×10^4 M^{-1} cm^{-1} at 359 nm for the acetic acid derivative of NCP in ethanol (Mitra & Hammes, 1989), 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 NBD-MANC was determined to be 2.12×10^4 M^{-1} cm^{-1} at 484 nm in methanol and 9.0×10^3 M^{-1} cm^{-1} at 480 nm in sonicated vesicles. Labeling stoichiometries were calculated after corrections for light scattering due to vesicles and protein.

Spectroscopic Measurements. Absorbance measurements were made with Cary 118 and Cary 2200 spectrophotometers. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. Fluorescence measurements on NCP-labeled protein were carried out at 4 °C except for one experiment at 20 °C; for NBD-labeled vesicles, fluorescence was recorded at room temperature. ESR spectra were obtained with a Bruker ER-200D-SRC spectrometer.

Results

Fluorescence Quenching by Spin-Labels. The use of doxylstearic acids in which the spin-label is placed at various positions along the fatty acyl chain to probe the depth of fluorescent species inside membranes is well documented (London & Feigensen, 1981; Blatt et al., 1984). In this study, stearic acids labeled with the doxyl group at positions 5, 7, 12, and 16 on the fatty acyl chain were employed to study the quenching of the fluorescence of NCP on the DCCD site of CF_1CF_o.

The decrease in fluorescence due to collisional (Stern–Volmer) quenching is given by (Lacowicz, 1983)

$$F_o/F = 1 + K_D[Q] \quad (1)$$

where F_o and F are the fluorescence intensities in the absence and presence of quencher, K_D is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher. The quenching observed in the experiments reported here is undoubtedly due to static quenching where a simple relationship such as eq 1 does not exist. Nevertheless, this equation provides a useful empirical basis for examining the results obtained. Figure 1 shows a plot of $F_o/F - 1$ for NCP-labeled CF_1CF_o vs quencher concentrations for the various doxylstearic acids used. The order of quenching efficiency is 5-SA > 7-SA > 12-SA ~ 16-SA. This implies that the NCP label in the membrane, and hence the DCCD site, is closest to 5-SA. If the depth of the doxyl groups inside the vesicles is assumed to be the same as the depth of the corresponding carbon atoms of the unlabeled fatty acid, then the 5-, 7-, 12-, and 16-doxyl groups are 6.25, 8.75, 15.0, and 20.0 Å from the outer surface of the vesicles (Voges et al., 1987; Dalton et al., 1987). The distance from the inner surface can be calculated by assuming a width of 37 Å for the bilayer (Huang & Mason, 1978).

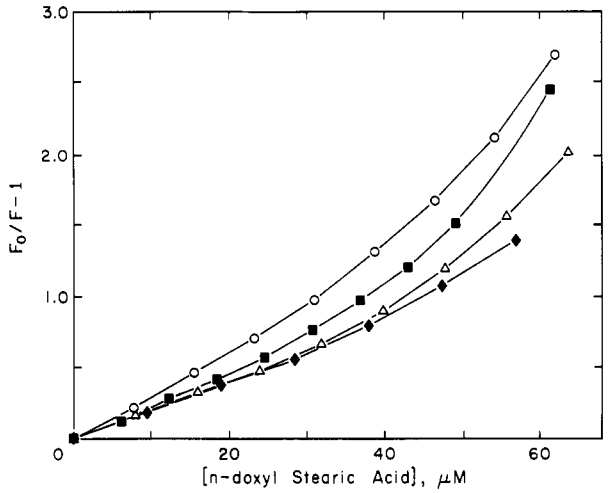

FIGURE 1: Plots of the fluorescence quenching, $F_0/F - 1$, of reconstituted NCP-CF_1CF_0 in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA vs the concentrations of spin-labels. F is the observed fluorescence and F_0 the fluorescence in the absence of spin-labels. The spin-labels used were 5- (○), 7- (■), 12- (△), and 16-SA (♦). The fluorescence was recorded at 375 nm (excitation 343 nm).

Since 5-SA is the most effective quencher of the NCP-labeled protein fluorescence, the DCCD site is approximately 6 Å or less from the vesicle surface.

To assess the depth of the NBD group on the two NBD-labeled lipids, NBD-PE and NBD-MANC, incorporated into asolectin vesicles, similar quenching experiments were performed. The decrease in fluorescence intensity of the NBD-labeled lipids in vesicles was recorded as a function of increasing concentrations of doxylstearic acids. Panels A and B of Figure 2 are plots of the quenching efficiency vs quencher concentration for NBD-PE and NBD-MANC, respectively. For NBD-PE the order of quenching is 5-SA > 7-SA ≫ 12-SA > 16-SA, while for NBD-MANC the quenching efficiency is 16-SA ~ 12-SA > 7-SA > 5-SA. This order of quenching places the NBD group in NBD-PE near the membrane surface, whereas the NBD group in NBD-MANC appears to be located at a depth of ~16 Å from the vesicle surface, i.e., at about the center of the bilayer. This result is in agreement with that of Chattopadhyay and London (1987), who measured the depths of various NBD-labeled lipids in multilamellar vesicles.

The fluorescence quenching experiments were performed in two ways. The spin-labels were either directly added to the vesicle samples containing the fluorophore, and the intensity recorded after ~5-min equilibration, or the spin-labels were codried with the lipids and sonicated to form unilamellar vesicles. In both cases, the quenching profiles generated were essentially the same. The NCP fluorescence quenching experiment was done at two temperatures, 4 and 20 °C. Within the experimental uncertainties, no significant difference in the degree of quenching at the two temperatures was observed.

Energy Transfer Measurements. For energy transfer measurements, the γ-disulfide site on CF_1CF_0 was labeled with the energy donor, CPM. Stoichiometries of labeling were generally kept less than 1.0 mol of CPM/mol of enzyme. Previous studies have shown that both sulfhydryl groups derived from the disulfide can be labeled (Snyder & Hammes, 1985). The extent of energy transfer is directly proportional to the labeling stoichiometry from <1 to ~2 labels/enzyme (mol/mol). Furthermore, when pyrenylmaleimide is the label, eximer fluorescence is seen at high stoichiometries, indicating the two sulfhydryls are within a few angstroms of each other. By keeping the stoichiometry relatively low, specificity is assured. The measured distance is not significantly affected because CPM is a donor and because of the proportionality discussed above.

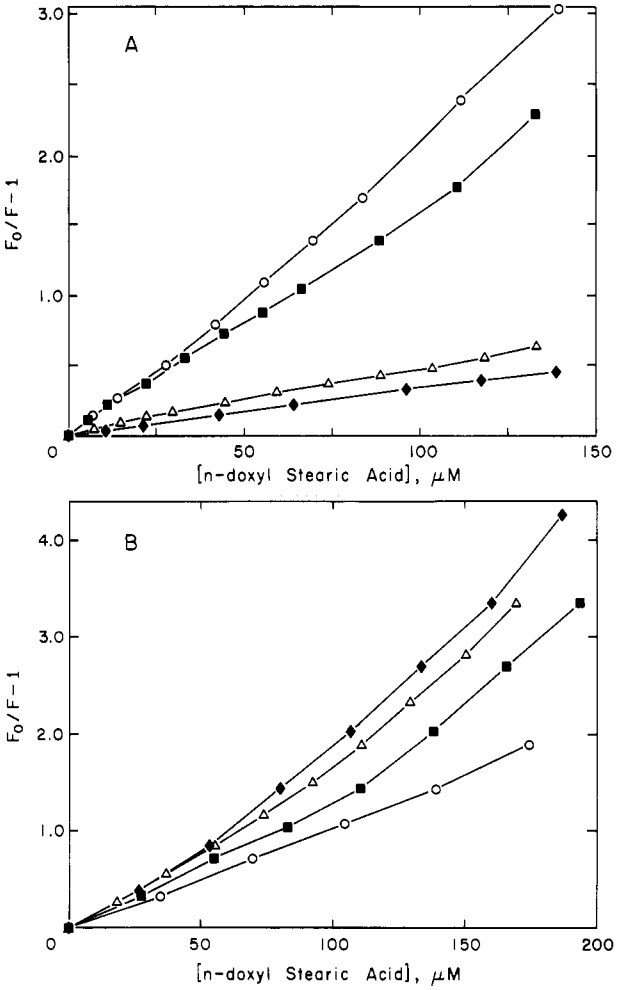

FIGURE 2: Plots of the fluorescence quenching, $F_0/F - 1$, of NBD-PE (A) and NBD-MANC (B) incorporated in asolectin vesicles vs the concentrations of spin-labels. F is the observed fluorescence and F_0 the fluorescence in the absence of spin-labels. The fluorescence was recorded at 530 nm (excitation 465 nm) in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA at room temperature. The spin-labels used were 5- (○), 7- (■), 12- (△), and 16-SA (♦).

The DCCD site was labeled with the energy donor NCP. The stoichiometry of [NCP]/[CF_1CF_0] was ~1.0 mol/mol. Previous work has shown that the loss of enzymatic activity is complete with a labeling stoichiometry of 1.0 mol/mol (Mitra & Hammes, 1989). This suggests that a unique site has been labeled with little nonspecific labeling.

For both the NCP- and CPM-labeled enzymes, the polarization is ≥0.2 (Table I), which indicates an environment of restricted rotation. Similar results have been found for other fluorescent labels on this enzyme [cf. Snyder and Hammes (1984, 1985)], and rates of rotation predominantly characteristic of the motion of the entire molecule have been found with dynamic fluorescence (Schinkel & Hammes, 1986) and phosphorescence (Musier-Forsyth & Hammes, 1990) measurements.

The labeled enzymes were purified on a sucrose gradient and concentrated with Sephadex G-25 medium when needed (Cerione & Hammes, 1981). The labeled enzyme was reconstituted with the octyl glucoside dilution procedure into vesicles labeled with the energy acceptor, NBD-MANC.

Figure 3 shows the fluorescence emission spectra of CPM-labeled protein and NCP-labeled protein and the absorbance

Table I: Energy Transfer Parameters for CF_1CF_o[a]

donor	fluorescence max (nm)	Q_D	P_D	acceptor	P_A	absorbance max (nm)	R_0 (Å)	L (Å)	D (Å)
NCP(DCCD)	375, 387	0.20	0.20	NBD-MANC(ves)	0.14	480	23.2	16	10
CPM(DiSH)	465	0.74	0.29	NBD-MANC(ves)	0.14		39.6	43	16

[a] Abbreviations: NCP(DCCD), NCP reacted at the DCCD site; CPM(DiSH), CPM label at the γ-disulfide site; NBD-MANC(ves), NBD-MANC distributed in the interior of the vesicle; P_D and P_A, steady-state polarization measured at fluorescence excitation and emission maxima of donor and acceptor, respectively; Q_D, quantum yield of the donor; L, distance of closest approach; D, perpendicular distance from the membrane surface. R_0 was calculated with eq 3.

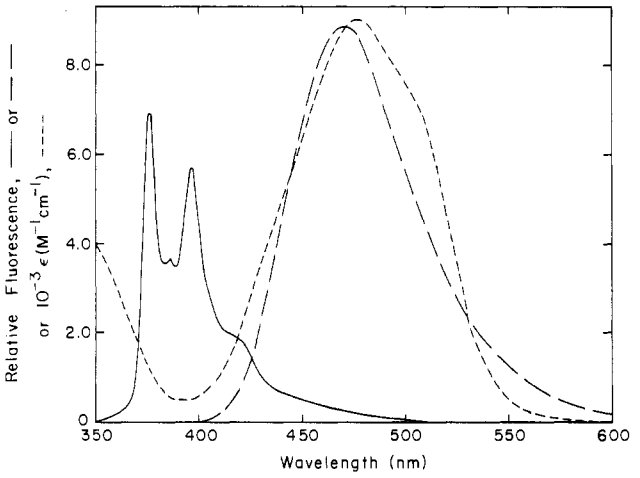

FIGURE 3: Spectral overlap of the fluorescence emission for NCP (--) bound to CF_o and for CPM (---) at the γ-disulfide site with the extinction coefficient (ε) of NBD-MANC in the vesicle (—). The fluorescence excitation was at 343 nm for NCP and at 387 nm for CPM. All spectra were taken in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA at room temperature.

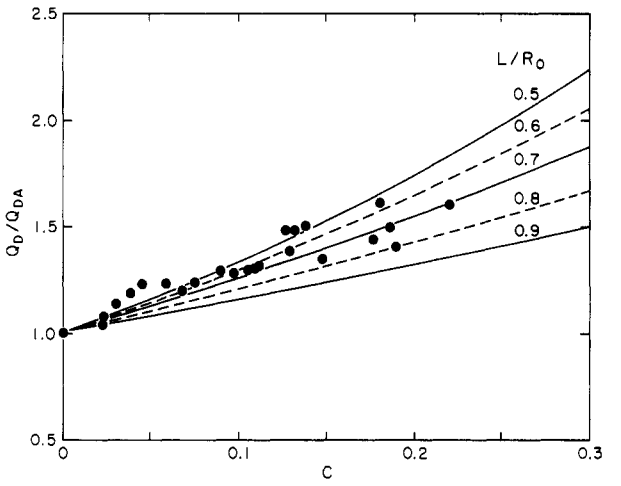

FIGURE 4: Plot of the ratio of the donor quantum yield in the absence and presence of the acceptor (NBD-MANC), Q_D/Q_{DA}, vs the normalized acceptor concentration, $C = \sigma R_0^2$, for reconstituted NCP-labeled CF_1CF_o. The fluorescence measurements were made in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA. The fluorescence excitation was at 343 nm, and the emission was at 375 nm. The curves represent the quenching expected for the given values of L/R_0 as calculated by Monte-Carlo simulations.

spectrum of NBD-MANC in vesicles. The extinction coefficient of NBD-MANC decreases by ~50% when it is incorporated into vesicles. The steady-state polarization of NBD-MANC in vesicles is 0.14, whereas that of NBD-PE is 0.12.

The two sulfhydryls of the reduced γ-disulfide were assumed to remain in close proximity (Snyder & Hammes, 1984). Hence, the energy transfer results were analyzed in terms of a single donor in one plane and a uniform distribution of acceptors on a second plane in the interior of the vesicles. Energy transfer measurements between the NCP-CF_1CF_o and the NBD-MANC vesicles also were analyzed in terms of this model.

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)

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

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

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

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

Here κ^2 is an 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 2 can be solved numerically. Several approximate solutions also have been developed (Dewey & Hammes, 1981; Wolber & Hudson, 1979). An approximate series solution to the integration is

$$Q_D/Q_{DA} = 1 + [(\pi\sigma R_0^2)/2][R_0/L]^4 \quad (4)$$

which is valid only for small extents of energy transfer. Snyder and Freire (1982) have presented theoretical quenching profiles obtained through Monte-Carlo simulations. The distance of closest approach, L, can be calculated by fitting the experimental data to these theoretical curves.

The density of acceptor molecules, σ, was calculated by assuming a uniform distribution of NBD-MANC at the center of the bilayer. A molecular weight of 564 was used for NBD-MANC. For asolectin, an average molecular weight of 578 was assumed (Sigma Chemical Co.). The vesicles were assumed to have a radius of 1000 Å and a bilayer width of 37 Å (Huang & Mason, 1978). R_0 was calculated with eq 3, and J was calculated from the spectra in Figure 3.

The ratio Q_D/Q_{DA} for CPM label at the γ-disulfide site is shown as a function of C ($=\sigma R_0^2$) (Figure 4). The curves represent Monte-Carlo solutions for the given values of L/R_0. The linear approximation, eq 4, gives $L/R_0 = 1.10$ and $L = 43.6$ Å, whereas the fit of the data to the Monte-Carlo simulations gives $L/R_0 = 1.08$ and $L = 42.8$ Å. The best fit of the data to the Monte-Carlo simulations was obtained by minimizing the root mean square deviations of the experimental points from the theoretical curves.

Figure 5 shows the plot of the quenching ratio for NCP-labeled protein vs C. The curves are Monte-Carlo solutions. Minimization of the root mean square deviation of the data

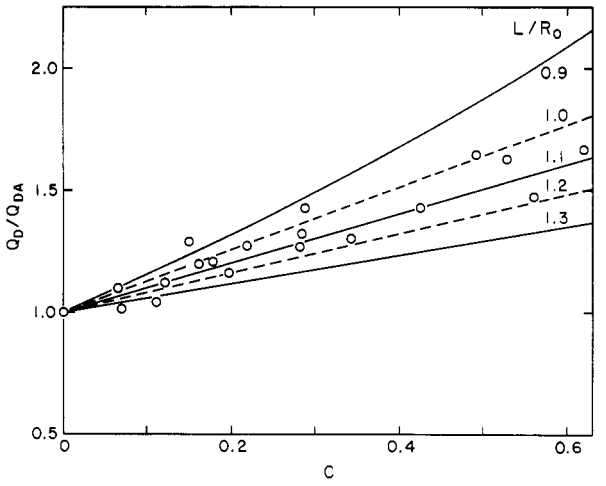

FIGURE 5: Plot of the ratio of the donor quantum yield in the absence and presence of the acceptor (NBD-MANC), Q_D/Q_{DA}, vs the normalized acceptor concentration, $C = \sigma R_0^2$, for CPM at the γ-disulfide site. The fluorescence measurements were made in 20 mM Na-Tricine (pH 8.0) and 1 mM EDTA. The fluorescence excitation was at 387 nm, and the emission was at 465 nm. The curves represent the quenching expected for the given values of L/R_0 calculated by Monte-Carlo simulations.

from the theoretical curves gives $L/R_0 = 0.65$ and $L = 15.1$ Å. The liner approximation gives $L = 20.3$ Å. The energy transfer results are summarized in Table I.

Discussion

Fluorescence resonance energy transfer techniques have been previously used to obtain information about the distance of closest approach of a fluorophore at a specific site on a protein to the surface of a membrane. Various sites on CF_1CF_0 have been specifically labeled with fluorescent molecules, and the distances of closest approach of these sites to the vesicle surface in reconstituted systems have been measured (Cerione et al., 1983; Snyder & Hammes, 1984; Mitra & Hammes, 1989). However, these studies do not provide information about the perpendicular distances between these sites and the membrane surface. In this work, a combination of fluorescence resonance energy transfer and spin-quenching techniques is used to measure the perpendicular distance between two specific sites on CF_1CF_0 and the membrane surface in a reconstituted system. The two specific sites are the disulfide site on the γ-polypeptide and the DCCD-reactive site on the c polypeptide.

Fluorescence quenching by spin-labels can be used to estimate the depth of a membrane-imbedded fluorophore. The doxylstearic acids, with the spin-label at different positions along the stearic acid chain, were used to generate quenching profiles for the NCP-CF_1CF_0 fluorescence. As shown in Figure 1, the quenching efficiency is greatest for 5-SA and least for 16-SA. This places the DCCD site closer to the membrane surface than to the center of the bilayer. This quenching profile is in contrast to the quenching pattern observed for the bovine mitochondrial proton channel, labeled with fluorescent analogues of DCCD, where 16-SA was observed to be the most effective quencher and 5-SA the least (Pringle & Taber, 1984), and it was concluded that the DCCD-binding sites were located at a depth of 18 Å from the membrane surface.

As a control experiment, similar quenching experiments were performed with the fluorescent lipid analogues, NBD-PE and NBD-MANC, incorporated into asolectin vesicles. Chattopadhyay and London (1987) performed similar experiments with NBD-labeled lipids inserted into pure phospholipid multilamellar vesicles. They concluded that the NBD group in NBD-PE was close to the membrane surface, ~4 Å deep, whereas the NBD label in NBD-MANC was anchored deep in the interior, ~13 Å from the surface. Our results, shown in Figure 2, are in agreement with this quenching pattern, except that the NBD group in NBD-PE appears to be even closer to the surface than 4 Å. This is because even though 5- and 7-SA are effective quenchers of NBD-PE fluorescence, 12- and 16-SA appear to be extremely poor quenchers, probably because they are further away than the critical separation distance of 11–12 Å required for collisional quenching (London & Feigensen, 1981).

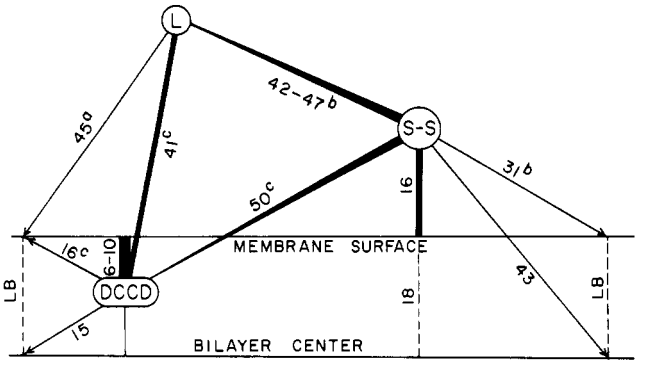

FIGURE 6: Model of the spatial relationships between the DCCD-binding site, the γ-disulfide site, and the membrane surface suggested by the energy transfer and spin-quenching measurements. The sulfhydryl site, L, on the γ-polypeptide is also shown. The distances are in angstroms, and LB represents the lateral boundaries of CF_1CF_0. (a) Cerione et al. (1983) with corrections for inner-filter effects; (b) Snyder and Hammes (1984); (c) Mitra and Hammes (1989).

Resonance energy transfer measurements have been made previously between the CPM-labeled γ-disulfide site and the vesicle surface labeled with NBD-PE (Snyder & Hammes, 1984) and between the NCP-labeled DCCD site and the NBD-PE-labeled vesicle surface (Mitra & Hammes, 1989). In this study, energy transfer measurements were performed with the CPM-labeled γ-disulfide site and the NCP-labeled DCCD site serving as energy donors. The energy acceptor used was NBD-MANC, distributed in the interior of the vesicle bilayer. To facilitate interpretation of the energy transfer data, the assumption was made that the entire concentration of NBD-MANC is uniformly distributed on a plane exactly in the center of the bilayer, 18 Å from the surface. This assumption is unlikely to introduce a large error since at worst NBD-MANC is distributed over a range of a few angstroms near the center of the bilayer. The polarization of NBD-MANC in vesicles (0.14) indicates it is somewhat constrained in its motion.

A distance of closest approach of 43 Å was calculated between the γ-disulfide site and the center of the bilayer. The distance of closest approach of the γ-disulfide site to the vesicle surface, 31 Å, is known from previous studies (Snyder & Hammes, 1984). If the lateral boundary between the two NBD-labeled lipids, one at the bilayer surface and one at the middle of the bilayer, and CF_1CF_0 is assumed to be the same and perpendicular to the bilayer surface, then a perpendicular distance of 16 Å can be calculated between the CPM-labeled γ-disulfide site and the membrane surface (see Figure 6).

For the NCP-labeled DCCD site, a distance of closest approach of 15 Å was obtained to the center of the bilayer. The distance of closest approach of this site to the vesicle surface is 16–24 Å (Mitra & Hammes, 1989). The upper limit, 24 Å, was calculated with the assumption that the DCCD site is 16 Å from the outer surface of the vesicle. This maximizes the contribution of energy acceptors on the inner surface of

the vesicle to the quenching of NCP fluorescence. This is clearly an overestimation of the upper limit since the spin-quenching experiments indicate that the DCCD site is ~6 Å from the surface. Therefore, the distance of closest approach of the DCCD site to the surface is probably close to 16 Å. This distance, together with a distance of closest approach of 15 Å to the center of the bilayer, would place the DCCD site at a vertical depth of ~10 Å from the membrane surface, assuming the same type of lateral boundary as discussed above. The vertical depth of the NCP-labeled site estimated from spin-quenching experiments, ~6 Å, is in reasonable agreement with the energy transfer results.

The vertical distances between two specific sites on CF_1CF_o and the membrane surface estimated in this study, together with previously mapped distances, are summarized in the model shown in Figure 6. Included are some of the distances associated with a sulfhydryl site on the γ-polypeptide (*L*). In this model, the DCCD site and the γ-disulfide site are on different sides of the molecule, and the DCCD site is relatively close to the surface. This model must be considered very approximate. In addition to the uncertainties in the measured distances, ±10% to ±20%, the assumption that the distances of closest approach to the vesicle surface and the middle of the bilayer are defined by the same lateral boundary (Figure 6) is particularly suspect.

The model used for determining the distances of closest approach assumes a symmetrical distribution of energy acceptors around the energy donor. Since the donor sites are asymmetrically placed in the protein, this is not quite correct. In terms of eq 2, this would reduce the factor of 2π. Reduction by a factor of 0.5, which is probably an overestimate of the effect of this asymmetry, would reduce *L* by a factor of approximately $2^{-1/4}$ (eq 4).

The fluorescent probes are large, can be oriented in specific ways, and have some mobility, as indicated by polarization measurements. This creates an uncertainty as to where the spectral probes are located relative to the specific sites: typically the uncertainty is about ±10 Å. Nevertheless, the probes define a specific point in space: in cases where significant motion occurs, this point would be a dynamic average. The protein probes are probably confined to a specific binding site, whereas the NBD-MANC can be defined by a region near the middle of the bilayer. This causes an uncertainty of about ±5 Å in how deep the enzyme model should be inserted into the membrane. However, the experiments with spin-labels are consistent with the results obtained from fluorescent resonant energy transfer and with the placement of NBD-MANC near the center of the bilayer.

In spite of the above uncertainties, a self-consistent model can be generated from a large number of different measurements. This model provides a starting point for further investigation.

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