Structural Mapping of Chloroplast Coupling Factor†

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ABSTRACT: Fluorescence resonance energy transfer measurements have been used to investigate the spatial relationships between the nucleotide binding sites and the γ-subunit of the H^+-ATPase from chloroplasts and the orientation of these sites with respect to the membrane surface. Fluorescent maleimides reacted covalently at specific sulfhydryl sites on the γ-subunit served as energy donors. One sulfhydryl site can be labeled only under energized conditions on the thylakoid membrane surface (light site). The two γ-sulfhydryls exposed after catalytic activation served as a second donor site (disulfide site). In one set of experiments, the nucleotide analogue 2′(3′)-(trinitrophenyl)adenosine triphosphate, selectively bound at each of the three nucleotide binding sites of the solubilized coupling factor, was used as an energy acceptor; in another, octadecylrhodamine with its acyl chain inserted in the vesicle bilayer and the rhodamine fluorophore exposed along the membrane surface was the energy acceptor. The distance between the sulfhydryl and disulfide sites was also obtained by sequentially labeling the sites with coumarin (donor) and fluorescein (acceptor) maleimide derivatives, respectively. The results indicate that all three nucleotide sites are ≈50 Å from the light-labeled γ-sulfhydryl. Two of the nucleotide sites are very far from the γ-disulfide (>74 Å), while the third site, which binds nucleotides reversibly under all conditions, is 62 Å from this sulfhydryl. The light-labeled sulfhydryl and disulfide sites are about 42–47 Å apart. Finally, the distance of closest approach between the membrane surface of the reconstituted system and the γ-disulfide is 31 Å. (All distances were calculated by assuming the donor and acceptor rotate freely and rapidly relative to the fluorescence lifetime of the donor.) These results have been used to construct a model of the overall structural characteristics of the coupling factor complex.

The dicyclohexylcarbodiimide-sensitive H^+-ATPase (DSA)[1] from spinach chloroplasts catalyzes ATP synthesis through partial dissipation of a proton gradient across the thylakoid membrane. The extrinsic portion of this membrane–enzyme complex, CF_1, is the site of nucleotide binding and catalysis and consists of five different types of polypeptides (α, β, γ, δ, ε; Pick & Racker, 1979) with a total molecular weight of 400 000 (Moroney et al., 1983). The polypeptide chain stoichiometry of the three largest polypeptides is probably $\alpha_3\beta_3\gamma$ (Moroney et al., 1983). CF_1 can be stripped from the thylakoid membrane through treatment with EDTA and when isolated forms a latent Ca^{2+}-specific ATPase (Farron & Racker, 1970; Lien & Racker, 1971). The intact DSA complex, on the other hand, can be solubilized through mild treatment with anionic detergents (Pick & Racker, 1979; Cerione & Hammes, 1981). This complex catalyzes P_i–ATP exchange when reconstituted in asolectin vesicles (Winget et al., 1977; Pick & Racker, 1979) and ATP synthesis when reconstituted with bacteriorhodopsin, a light-induced proton pump (Winget et al., 1977; Dewey & Hammes, 1981; Takabe & Hammes, 1981). Both enzyme systems can be readily activated through reduction of a cysteine disulfide on the γ-subunit of CF_1 (Farron & Racker, 1970; Pick & Racker, 1979).

Previously three distinct nucleotide binding sites have been characterized on solubilized CF_1 (Bruist & Hammes, 1981) and the intact DSA complex (Cerione & Hammes, 1981). For CF_1, the site designated as site 1 contains tightly bound ATP which cannot be removed by extensive dialysis but exchanges readily with medium nucleotides; site 2 binds ATP tightly in the presence of Mg^{2+}; site 3 binds ATP and ADP reversibly under all conditions with dissociation constants in the micromolar range. The use of nucleotide analogues as photoaffinity reagents has shown that photoaffinity labeling of site 1 primarily modifies the β-polypeptide chain (N. Kambouris and G. G. Hammes, unpublished results), labeling of site 2 primarily modifies the β-polypeptide chain, and labeling of site 3 modifies both the α- and β-polypeptide chains approximately equally (Bruist & Hammes, 1981). Fluorescence resonance energy transfer studies with nucleotide analogues have shown that the three nucleotide binding sites form a roughly symmetrical triangle with separation distances of approximately 40 Å (Cerione & Hammes, 1982).

Four cysteinyl residues have been characterized on the γ-subunit of CF_1 on the basis of their reactivity with various alkylating reagents (Nalin & McCarty, 1984; Moroney et al., 1984). Two of these sulfhydryls can be distinguished by the dependence of their reactivity on the energy state of the thylakoid membrane; i.e., one can be modified either in the dark or in the light (dark site), while the other is accessible only in the light (light site). Labeling of the light site results in inhibition of photophosphorylation. The two remaining γ-sulfhydryls are those exposed after reduction of the disulfide bond during catalytic activation. Each of these sulfhydryls can be readily labeled with several strongly absorbing, highly fluorescent maleimide derivatives (Cantley & Hammes, 1976; Nalin et al., 1983; Cerione et al., 1983; Nalin & McCarty, 1984). In the present study, the spatial relationships between the nucleotide binding sites of CF_1, the γ-sulfhydryls described above, and the membrane surface of the reconstituted system are investigated by using fluorescence energy transfer techniques. The results of these studies can be used to create a

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[1] Abbreviations: DSA, dicyclohexylcarbodiimide-sensitive H^+-ATPase; CF_1, chloroplast coupling factor 1; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; TNP-ATP, 2′(3′)-(trinitrophenyl)adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]]maleimide; DMSM, *N*-(2,5-dimethoxystilben-4-yl)maleimide.

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working model for overall structural characteristics of the DSA–enzyme complex.

Materials and Methods

Chemicals. Octyl glucoside was purchased from Calbiochem. Cholic acid, recrystallized prior to use (Kagawa & Racker, 1971), ATP (vanadium free), and Triton X-100 were from Sigma Chemical Co. Asolection (crude soybean phospholipids) was obtained from Associated Concentrates, Woodside, NY, and was used without further purification. [^{3}H]ATP was from New England Nuclear, [^{32}P]P_i was from ICN and was heated to 90 °C in 2 N HCl for 2 h to hydrolyze any pyrophosphate which might be present. TNP-ATP, NBD-PE, octadecylrhodamine, and all fluorescent maleimide derivatives were from Molecular Probes, Inc. Disodium fluorescein was obtained from Eastman Chemical Co. and quinine sulfate from Aldrich Chemical Co. 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 having a fluorescent emission ratio, 305 nm/340 nm (excitation 280 nm), greater than 1.5 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. The molar concentrations of CF_1 were determined by using an extinction coefficient of 0.483 mL/(mg·cm) at 277 mM (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 of at least 12 μmol/(mg·min) at 25 °C.

Covalent Labeling of CF_1. Prior to heat activation, exposed sulfhydryls were blocked by reacting CF_1 with 10 mM *N*-ethylmaleimide for 30 min in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA. Unreacted *N*-ethylmaleimide was removed by passing the solution through a 3-mL centrifuge column (Penefsky, 1977) equilibrated with the same buffer. The enzyme was then heat activated by adding ATP and dithiothreitol to final concentrations of 40 and 7 mM, respectively, and heating at 63–64 °C for 4 min. ATP and dithiothreitol were removed by passing the enzyme through two consecutive 3-mL centrifuge columns equilibrated with the above buffer. Finally, the two γ-subunit sulfhydryls exposed by disulfide reduction during heat activation (Cantley & Hammes, 1976) were labeled by adding a 50 μM sample of the chosen fluorescent maleimide derivative and reacting for 5 min before passing the mixture through two to three consecutive 3-mL centrifuge columns to remove unreacted probe.

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), 7.08×10^4 M^{-1} cm^{-1} at 495 nm for fluoresceinylmaleimide (Cerione et al., 1983) 3.02×10^4 M^{-1} cm^{-1} at 387 nm for CPM (Sippel, 1981), and 1.40×10^4 M^{-1} cm^{-1} at 344 nm for DMSM determined for the dithiothreitol-conjugated moiety in absolute ethanol. Corrections were made for probe absorbance at 277 nm and CF_1 light scattering at the absorption maxima. Stoichiometries of covalently bound label for the given reaction conditions were in the range of 0.7–1.0 mol of probe/mol of CF_1 except for pyrenylmaleimide where, due to its extreme hydrophobicity, stoichiometries approached 1.5–2.0 mol/mol. Specificities for the γ-subunit were >95% as visualized by the fluorescence of sodium dodecyl sulfate–polyacrylamide slab gels illuminated with near-ultraviolet light (Weber & Osborne, 1969).

Labeling of the buried γ-sulfhydryl of CF_1 on thylakoids under energized conditions and subsequent isolation of the solubilized CF_1 complex were performed as described previously (Cerione et al., 1983; Nalin et al., 1983). For these labeling experiments, the concentration of CPM was 50 μM, and labeling stoichiometries were 0.6–0.7 mol of CPM/mol of CF_1. Only a single fluorescent band corresponding to the γ-subunit of CF_1 was observed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified enzyme sample.

Labeling of Nucleotide Sites on CF_1. Specific labeling of the nucleotide site which binds ADP very tightly but exchanges readily with medium nucleotides (nucleotide site 1; Bruist & Hammes, 1981) was obtained by incubating either the labeled heat-activated CF_1 or the light-labeled CF_1 (1–5 μM) with 50–100 μM TNP-ATP for 1–3 h in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA. Unbound and dissociable nucleotides were then removed by passing the incubation mixture through one or two consecutive 3-mL centrifuge columns equilibrated with the same buffer. Under these conditions, binding at all other nucleotide sites on CF_1 was reversible. TNP-ATP binding stoichiometries were calculated by using an extinction coefficient of 2.51×10^4 M^{-1} cm^{-1} at 418 nm for the bound nucleotide (Cerione & Hammes, 1981), after correcting for probe absorbance at 277 nm and CF_1 light scattering at 418 nm. The final binding stoichiometries were 1.0–1.1 mol of TNP-ATP/mol of CF_1. Calculated stoichiometries in excess of equimolar may be due to the loss of smaller subunits during repeated column centrifugation, which could result in an underestimate of the molar concentration of CF_1.

Simultaneous labeling of nucleotide site 1 and the site which binds MgATP very tightly (nucleotide site 2; Bruist & Hammes, 1981) on the light-labeled CF_1 was obtained by incubating the enzyme (1–3 μM) with 50–100 μM TNP-ATP in 6 mM $MgCl_2$, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA. Free and dissociable nucleotides were again removed by column centrifugation. For this case, labeling stoichiometries were in the range of 2.0–2.2 mol of TNP-ATP/mol of CF_1, twice those obtained for site 1 labeling only. For the heat-activated enzyme, binding of MgTNP-ATP at site 2 was not tight; however, tight binding of 2.0–2.2 mol of [^{3}H]ATP/mol of CF_1 could be obtained in a manner similar to that described above. After incubation with excess TNP-ATP and removal of free and dissociable nucleotides by column centrifugation, final labeling stoichiometries of 1.0–1.1 mol of TNP-ATP at site 1 and 1.0–1.1 mol of [^{3}H]ATP at site 2/mol of CF_1 could be obtained. In this manner, site 2 on the maleimide-labeled heat-activated enzyme could be blocked from further binding. Similarly, addition of excess ATP to the light-labeled latent CF_1 containing both site 1 and site 2 bound TNP-ATP could be used to obtain specific TNP-ATP labeling of nucleotide site 2.

Preparation of Maleimide-Labeled DSA. Partially purified DSA was prepared from fresh market spinach by a modification of the procedure of Pick & Racker (1979; Cerione & Hammes, 1981; Cerione et al., 1983). One extra wash of the thylakoid suspension with 10 mM NaCl was included to remove further the ribulose-1,5-bisphosphate carboxylase contaminant. Protein concentrations were determined according to Bensadoun & Weinstein (1976) with bovine serum albumin as a standard. A molecular weight for DSA of 500 000 was assumed for calculation of molar concentrations. The partially

purified DSA was assayed for [^{32}P]P_i–ATP exchange activity as described previously (Cerione et al., 1983). Specific activities were in the range 80–120 nmol of P_i/(mg·min) (37 °C). All incubations and further purifications of DSA were performed at 4 °C.

To specifically label the γ-subunit of DSA, the partially purified enzyme was first incubated with 10 mM *N*-ethylmaleimide for 30 min in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA to block exposed sulfhydryls and passed through one 3-mL centrifuge column equilibrated with the same buffer to remove unreacted *N*-ethylmaleimide. The enzyme was then incubated with 50 mM dithiothreitol for 1 h to reduce the disulfide on the γ-subunit of DSA. This disulfide is the same as that reduced during heat activation of the solubilized CF_1 complex (Ketcham et al., 1984). After removal of the dithiothreitol by passage through two consecutive centrifuge columns equilibrated with the above buffer, either fluoresceinylmaleimide or CPM was added to a final concentration of 50 μM and reacted for 5 min before removal of the unreacted probe by column centrifugation. The modified crude enzyme was then further purified by sucrose density centrifugation and subsequent elution through a Sepharose 4B–anti-ribulosebisphophate carboxylase column (0.8 mL) as described previously (Cerione et al., 1983). When this procedure was used, a specific labeling of the γ-subunit of DSA was obtained with essentially complete removal of pigment and ribulosebisphosphate carboxylase contaminants as visualized by sodium dodecyl sulfate–gel electrophoresis. Final exchange activities were ≈10–30% those of the original partially purified enzyme mixture. The labeling stoichiometries for both probes were ≈0.5 mol/mol of DSA.

Preparation of Reconstituted DSA for Energy Transfer Measurements. Vesicles containing NBD-PE or octadecylrhodamine (energy acceptors) were prepared by adding 0.05–0.2 mg of these compounds in 50 μL of dimethyl sulfoxide to 0.5 mL of 40 mg/mL asolectin in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA. The samples were then sonicated to clarity in a bath-type sonication (≈5 min) and resonicated for 15 s just prior to use. The final eluted DSA samples from the Sepharose 4B–anti-ribulosebisphosphate carboxylase column were concentrated with Sephadex G-25 (medium) (Cerione & Hammes, 1981) and reconstituted by adding octyl glucoside (final concentration of 0.7%) to a 1:1 mixture of enzyme (0.2–0.4 mg/mL) and the acceptor containing asolectin vesicles. This solution was incubated on ice for 5 min, and then a 15-μL aliquot was diluted to 1 mL with 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA for use in energy transfer measurements. Maleimide–DSA samples reconstituted in asolectin vesicles containing 9% dimethyl sulfoxide served as energy transfer donors in the absnce of acceptor. Final acceptor concentrations in the vesicle solutions were calculated by using extinction coefficients of 2.24×10^4 M^{-1} cm^{-1} at 465 nm for NBD-PE and 9.54×10^4 M^{-1} cm^{-1} at 564 nm for octadecylrhodamine (Cerione et al., 1983) after correction for light scattering of the asolectin vesicles.

Spectroscopic Measurements. Absorption spectra were obtained 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. Quantum yields for the fluorescent-labeled CF_1 samples were determined by a comparative method (Parker & Reese, 1966) using

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

where Q_1/Q_2 is the ratio of the quantum yields, F_1/F_2 is the ratio of the areas for the corrected emission spectra, and A_2/A_1 is the ratio of the absorbances at the excitation wavelengths for each of the two fluorophores. Sodium fluorescein in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA and quinine sulfate in 0.1 N H_2SO_4 were used as standards and were assumed to have quantum yields of 0.92 (Weber & Teale, 1957) and 0.70 (Scott et al., 1970), respectively. The fluorescence emission for the modified CF_1 samples was corrected for light scattering by subtracting the apparent emission for an equal concentration of CF_1 alone. Due to the low protein concentrations and large amount of light scattering for the maleimide containing reconstituted DSA, quantum yields and corrected emission spectra for these samples were estimated from equivalently labeled heat-activated CF_1. Steady-state polarization measurements, with corrections for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating, were made as previously described (Cerione & Hammes, 1982).

Time-Resolved Fluorescence Measurements. Fluorescence lifetime measurements were made with an ORTEC 9200 nanosecond fluorescence spectrophotometer described previously (Matsumoto & Hammes, 1975). The CPM-labeled CF_1 samples were excited with light from a spark gap flash lamp that was vertically polarized and filtered through a Corning CS7-51 band-pass filter with cutoffs at 310 and 410 nm. The fluorescence emission was either vertically or horizontally polarized and passed through a Corning CS3-73 425-nm cutoff filter before entering the photomultiplier. Under the given experimental conditions, background and scattering corrections were negligible. The time-resolved fluorescence, $F(t)$, was then calculated as

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

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

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 stoichiometry of the acceptor binding. Efficiencies of energy transfer were 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. Distances between specific donor and acceptor sites were calculated by using

$$E = [\sum_{i=1}^{N_A}(R_0/R_i)^6]/[1 + \sum_{i=1}^{N_A}(R_0/R_i)^6] \quad (4)$$

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 4 assumes each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of CF_1. In most cases, only a single donor–acceptor pair was present; however, when multiple acceptors were present, all but one of the distances had been measured previously so that eq 4

Table I: Energy Transfer Parameters for CF_1 and DSA[a]

donor	location	emission max (nm)	Q_D	P^b	acceptor	location	absorption max (nm)	R_0^c (Å)
CPM	di-SH	470	0.74	0.29	TNP-ATP	N1–N3	418/480	45.2
DMSM	di-SH	420	0.17		TNP-ATP	N1	418/480	34.6
CPM	L	470	0.70	0.42	TNP-ATP	N1–N3	418/480	44.8
CPM	L	470	0.70	0.42	FM	di-SH	495	50.3
FM	di-SH	523	0.35	0.24	ODR	membrane	564	52.6
CPM	di-SH	470	0.74	0.29	NBD-PE	membrane	465	44.7

[a] di-SH represents the two γ-sulfhydryls exposed upon heat activation of CF_1 or dithiothreitol treatment of DSA, L is the γ-sulfhydryl labeled in the light on the thylakoids, N1–N3 are nucleotide binding sites 1–3 on CF_1, FM is fluoresceinylmaleimide, and ODR is octadecylrhodamine. [b] Steady-state polarization measured at fluorescence excitation and emission maxima. [c] R_0 calculated by using eq 5 and the spectral properties of the donor and acceptor molecules. The value of κ^2 is assumed to be $^2/_3$.

contained only one unknown. In this equation, R_0 is given by (Förster, 1959)

$$R_0 = (9.79 \times 10^3)(\kappa^2 J Q_D n^{-4})^{1/6} \quad (5)$$

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; however, when possible, upper and lower bounds for κ^2 were calculated from the observed polarizations of donor and acceptor molecules (Dale et al., 1979). The contributions of inner filter effects to the steady-state fluorescent measurements were determined from the observed decrease in fluorescence of either a dithiothreitol or an *N*-acetylcysteine donor adduct caused by an equal concentration of free acceptor molecule. Equivalently, these effects could be calculated directly from the measured acceptor absorbance at the donor excitation and emission wavelengths.

For the reconstituted DSA system, vesicle solutions containing an equivalent concentration of acceptor molecules, but no labeled enzyme, were used as scattering blanks, and the donor concentration was assumed to be the same in each sample. The surface density, σ, of acceptor molecules (number of acceptor molecules per square angstrom of phospholipid vesicle surface) was calculated by using the known molecular weights for the different acceptors and assuming a molecular weight of 740 for asolectin. The value of Huang & Mason (1978) for the average phospholipid molecular volume and bilayer width were used to calculate the average surface area per lipid molecule. The acceptor molecules were assumed to be uniformly distributed on the inner and outer layers of the vesicles with only acceptor molecules on the outer surface contributing to the observed energy transfer. Inner filter effects were calculated in a similar manner to those for the CF_1 energy transfer determinations.

Distances of closest approach, L, between the donor-labeled γ-subunit of DSA and energy acceptors uniformly distributed along the outer membrane surface were calculated by fitting the experimental quenching profiles to theoretical curves obtained through a method of Monte Carlo simulation (Snyder & Freire, 1982). These simulations were carried out for the case of two infinite planes separated by a distance L, one containing a random distribution of donors and the other a random distribution of acceptors. Under the given conditions

$$Q_D/Q_{DA} = N_D\{\sum_{j=1}^{N_D}[1 + \sum_{k=1}^{N_A}(R_0/R_{D_jA_k})^6]^{-1}\}^{-1} \quad (6)$$

where the sum j is over all donors, the sum k is over all acceptors, and $R_{D_jA_k}$ is the distance separating donor j and acceptor k. This situation is statistically equivalent to a single donor separated by a distance L from an infinite plane of randomly distributed acceptors, in which case the total number of donors, N_D, is equal to 1. For the calculation of experimental distances of closest approach, a set of theoretical curves for Q_D/Q_{DA} vs. the normalized acceptor density (σR_0^2) was created for a series of L/R_0 values within the range of the observed data. A best fit for L/R_0, and hence L, was obtained by minimizing the root mean square error between the theoretical curves and the observed quenching ratios.

Results

CF_1 γ-Disulfide as a Donor Site. Before fluorescence energy transfer measurements with the γ-disulfide-labeled CF_1 were performed, the proximity of the two sulfhydryls exposed after reduction of the disulfide during heat activation was checked by labeling each with the fluorescent probe pyrenylmaleimide. Most pyrene derivatives have the special property that when two such molecules are adjacent, with their dipole moments correctly aligned, they form an excimer which yields an emission fluorescence distinct from that of the monomeric species (Betcher-Lange & Lehrer, 1978; Angelides & Hammes, 1979). Given this property, labeling of the γ-disulfide with pyrenylmaleimide, where stoichiometries approach 2.0 mol of probe/mol of CF_1, and inspection of the resulting emission spectrum for excimer fluorescence provide a quick test of whether the labeled sulfhydryls remain in close proximity after the labeling procedure. After modification with pyrenylmaleimide, the emission fluorescence of the labeled CF_1 was found to contain about a 10% contribution from the excimer species. This result provides good evidence that the labeled sulfhydryls remain close after modification and that distance measurements with either sulfhydryl labeled should yield equivalent results.

Initial attempts at resonance energy transfer measurements with the γ-disulfide-labeled CF_1 were made with DMSM. Its fluorescence properties are summarized in Table I. The fluorescence emission spectrum of the modified CF_1 has an excellent overlap with the absorbance spectrum of TNP-ATP (Figure 1). After the heat-activated CF_1 was labeled with DMSM, TNP-ATP was incorporated at nucleotide site 1. The efficiency of energy transfer between the labeled γ-subunit and nucleotide site 1 was then measured by comparing the observed fluorescence for the CF_1 containing TNP-ATP to that containing no TNP-ATP, after normalizing with respect to the protein concentrations. No energy transfer could be detected within the resolution of the experiment ($E < 0.05$). Given the R_0 for this donor–acceptor pair (34.6 Å), the absence of detectable energy transfer suggests that the distance separating these two sites is >57 Å.

To obtain a larger R_0, the disulfide modification of CF_1 was repeated by substituting CPM for DMSM (see Table I and Figure 1 for the fluorescence properties of CPM). Even with the increased sensitivity of the CPM label (R_0 = 45.2 Å), no energy transfer could be detected between CPM on the disulfide and TNP-ATP in site 1. If an experimental error of

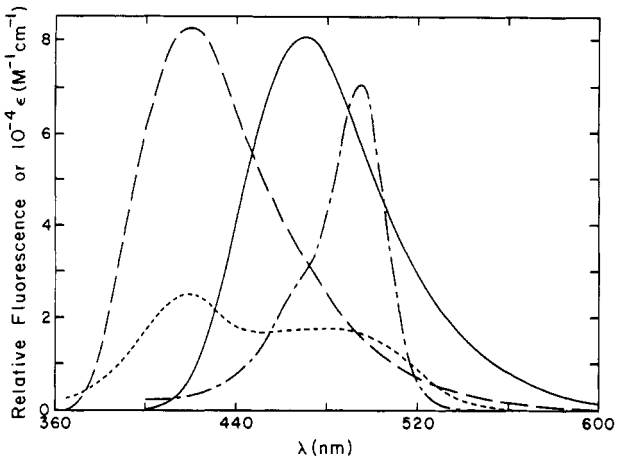

FIGURE 1: Spectral overlap of the fluorescence emission for CPM (—), covalently bound to CF_1 at either the sulfhydryl site labeled in the light or the disulfide site of the γ-subunit, and DMSM (– –), covalently bound to CF_1 at the γ-disulfide site, with the extinction coefficient (ε) of TNP-ATP (- - -), bound to the nucleotide sites of CF_1, and fluoresceinylmaleimide (–·–), covalently bound to the γ-disulfide site. The fluorescence excitation was at 387 nm for CF_1–CPM and 344 nm for CF_1–DMSM. All spectra were taken in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA at 25 °C.

5% is assumed, this result yields a separation of >74 Å.

Two further checks were made to test for evidence of energy transfer beteen the CPM-labeled γ-disulfide and nucleotide site 1 of CF_1. In the first, a large excess of ATP was added to the fluorescent CF_1 containing TNP-ATP at site 1. If energy transfer is occurring, the fluorescence should increase as the TNP-ATP is displaced by ATP. However, no change in fluorescence occurred. In the second experiment, time-resolved fluorescence was used to compare the fluorescence lifetimes of the sample containing TNP-ATP bound at site 1 to that containing no TNP-ATP. In both cases, the measured lifetime for the CPM adduct was the same, 3.9 ns.

Measurement of energy transfer from the CPM-labeled γ-disulfide of heat-activated CF_1 to nucleotide site 2 could not be obtained directly from relative fluorescence measurements since TNP-ATP dissociates within seconds. To measure the relative quenching of CPM by TNP-ATP at nucleotide site 2, the modified CF_1 containing TNP-ATP bound at site 1 was titrated with TNP-ATP, and the decrease in the steady-state fluorescence of the CPM label was monitored. To distinguish between quenching by binding at nucleotide site 2 and the site which binds nucleotides reversibly under all conditions (nucleotide site 3), the titration measurements were repeated with site 2 blocked by [^{3}H]ATP. The titration with site 2 unblocked was also performed by monitoring the time-resolved fluorescence of the CPM adduct. For the steady-state measurements, large inner filter corrections were required due to the presence of free TNP-ATP at concentrations >1 μM; these corrections were not necessary for the fluorescence lifetime measurements. The titrations are shown in Figure 2A as plots of the quantum yield and lifetime quenching ratios for CPM vs. the total concentration of TNP-ATP. The concentration of labeled CF_1 in these experiments was ≈0.1 μM. A gradual decrease in the quenching ratio occurs until it levels off at ≈10 μM TNP-ATP. Good agreement between the steady-state and time-resolved fluorescence measurements is observed. For concentrations of TNP-ATP >16 μM, significant quenching was observed due to nonspecific binding of the TNP-ATP probe (data not shown). The degree of quenching of the CPM label was found to be independent of whether site 2 was blocked with Mg-[^{3}H]ATP. Thus, nucleotide site 2, like site 1, appears to be very far from the γ-disulfide of CF_1 (R > 74 Å).

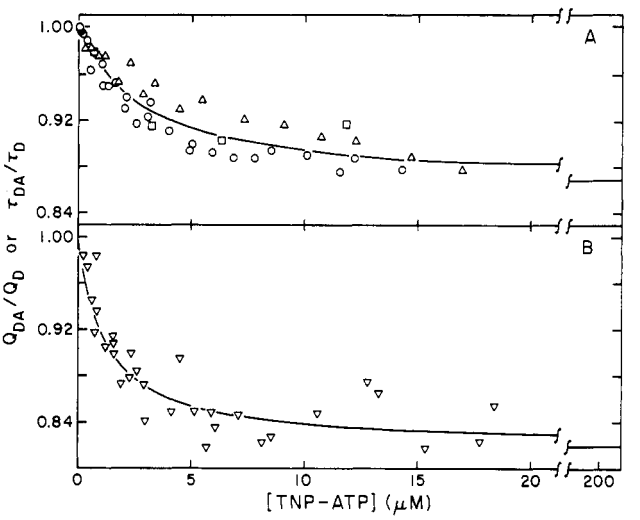

FIGURE 2: Plots of the fluorescence quenching of CPM bound to CF_1, Q_{DA}/Q_D or τ_{DA}/τ_D, vs. the total concentration of TNP-ATP. Here Q_D and Q_{DA} are the quantum yields in the absence and presence of TNP-ATP, respectively, and τ_D and τ_{DA} are the corresponding fluorescence lifetimes (387-nm excitation, 465-nm emission). (A) CPM covalently bound to the γ-disulfide of CF_1 and TNP-ATP bound to site 1 with (○) and without (△) [^{3}H]ATP bound to site 2 for Q_{DA}/Q_D and without [^{3}H]ATP (□) for τ_{DA}/τ_D. (B) Q_{DA}/Q_D for CPM covalently bound at the sulfhydryl labeled in the light (▽) with TNP-ATP bound at both site 1 and site 2. All titrations were performed in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 6 mM $MgCl_2$, and 2 mM EDTA at 25 °C. The curves are nonlinear least-squares fits of the data to eq 7 as described in the text.

Since the observed quenching appears to be entirely due to binding at nucleotide site 3, the titration data were fit by assuming the nucleotide binds to a single site on the enzyme. In this case

$$Q_{DA}/Q_D = \tau_{DA}/\tau_D = 1 - E[EL]/[E_0] \quad (7)$$

where E is the energy transfer efficiency when site 3 is fully occupied and [EL], the concentration of bound ligand, can be calculated from the dissociation constant, K_d, and the total concentrations of enzyme, $[E_0]$, and ligand, $[L_0]$, by using

$$2[EL]/[E_0] = (1 + [L_0]/[E_0] + K_d/[E_0]) - \{(1 + [L_0]/[E_0] + K_d/[E_0])^2 - 4[L_0]/[E_0]\}^{1/2} \quad (8)$$

A nonlinear least-squares fit of the data gives $K_d = 2.6 \pm 0.4$ μM, in good agreement with previously determined dissociation constants for the binding of nucleotides at site 3 (Cantley & Hammes, 1975; Bruist & Hammes, 1981; Cerione & Hammes, 1982), and $E = 0.13 \pm 0.01$. The energy transfer efficiency is independent of whether site 2 is blocked, but if the data for the case in which site 2 contains [^{3}H]ATP are fit separately, a slightly higher K_d, ≈4 μM, is obtained. An energy transfer efficiency of 0.13 corresponds to a separation of 62 Å between the two labeled sites.

CF_1 Light-Labeled γ-Sulfhydryl as a Donor Site. To maintain sensitivity for the measurement of longer distances, the labeling of the light-labeled γ-sulfhydryl of CF_1 was again performed with CPM. The fluorescence and energy transfer parameters for this label are included in Table I. The CPM adduct at this site had an equivalent corrected emission spectrum and a quantum yield within 5% of that of the CPM-labeled γ-disulfide. However, the steady-state polarization for this modified γ-sulfhydryl (0.42) was significantly higher than that for the γ-disulfide CPM (0.29). The intrinsic polarizations for both cases were not altered by binding at any of the three nucleotide sites.

The efficiency of energy transfer between the light-labeled γ-sulfhydryl and TNP-ATP bound at nucleotide site 1 was determined by using three independent techniques. In the first, the fluorescence intensity for the TNP-ATP-labeled CF_1 was compared to that containing no TNP-ATP. In the second, excess ATP was added to the TNP-ATP sample, and the increase in fluorescence caused by displacement of TNP-ATP bound at site 1 was monitored. Lastly, a concentration of TNP-ATP low enough to result in negligible binding at sites 2 and 3 but high enough to exchange with the majority of site 1 bound nucleotides was added to the light-labeled CF_1, and the decrease in the observed fluorescence was measured. All three determinations gave quenching ratios within 2% of the average and yielded an energy transfer efficiency of 0.30 per mol of bound TNP-ATP. This efficiency corresponds to a separation distance of 52 Å between the light-labeled γ-sulfhydryl and nucleotide site 1.

For the light-labeled enzyme, the binding of TNP-ATP at nucleotide site 2 in the presence of Mg^{2+} was found to be tight. For this reason, the efficiency of energy transfer between the buried γ-sulfhydryl modified with CPM and TNP-ATP bound at nucleotide site 2 could be measured directly, rather than by a titration. This was done by first saturating the light-labeled CF_1 with TNP-ATP at nucleotide sites 1 and 2. Comparison of the fluorescence of this sample to that containing no TNP-ATP yielded a transfer efficiency of 0.49. Excess ATP was then added to the TNP-ATP-containing enzyme to displace all TNP-ATP bound at site 1. This exchange resulted in an increase of 33% in the observed CPM fluorescence. From these two measurements, a transfer efficiency of 0.32/mol of TNP-ATP bound at site 2, corresponding to a separation distance of 51 Å, can be calculated. Alternatively, the distance from the buried γ-sulfhydryl to nucleotide site 2 can be calculated from eq 4 by using the observed transfer efficiency for the enzyme containing both site 1 and site 2 bound TNP-ATP and the known distance to nucleotide site 1. This technique yields an essentially identical distance, 50 Å.

The measurement of energy transfer between the light-labeled γ-sulfhydryl of CF_1 and nucleotide site 3 was performed by titrating the enzyme with TNP-ATP and monitoring the decrease in the steady-state fluorescence. To ensure that the observed quenching was due solely to binding at site 3, the light-labeled CF_1 was first saturated with TNP-ATP at nucleotide sites 1 and 2. The results of this titration are presented in Figure 2B. (No evidence of nonspecific binding of TNP-ATP at high probe concentrations was found.) A nonlinear least-squares fit of the data to eq 7 gives $K_d = 1.2 \pm 0.2$ μM and $E = 0.18 \pm 0.01$. The binding of TNP-ATP at nucleotide site 3 thus appears to be somewhat tighter for the light-labeled enzyme than for the labeled heat-activated CF_1. The total energy transfer efficiency for enzyme containing 1 mol of bound TNP-ATP at each of the three nucleotide sites is 0.58. By use of eq 4, the total transfer efficiency for binding at all three sites, and the distances calculated above for sites 1 and 2, a distance of 51 Å between the buried γ-sulfhydryl and nucleotide site 3 can be calculated.

To examine the spatial relationships between the two γ-sulfhydryl labeling sites described in this study, the light-labeled enzyme was heat activated in the presence of dithiothreitol and further modified with fluoresceinylmaleimide at the γ-disulfide site. The fluorescence and energy transfer parameters of fluoresceinylmaleimide at the disulfide site are summarized in Table I. Its absorption spectrum and spectral overlap with the emission fluorescence of the CPM adduct are

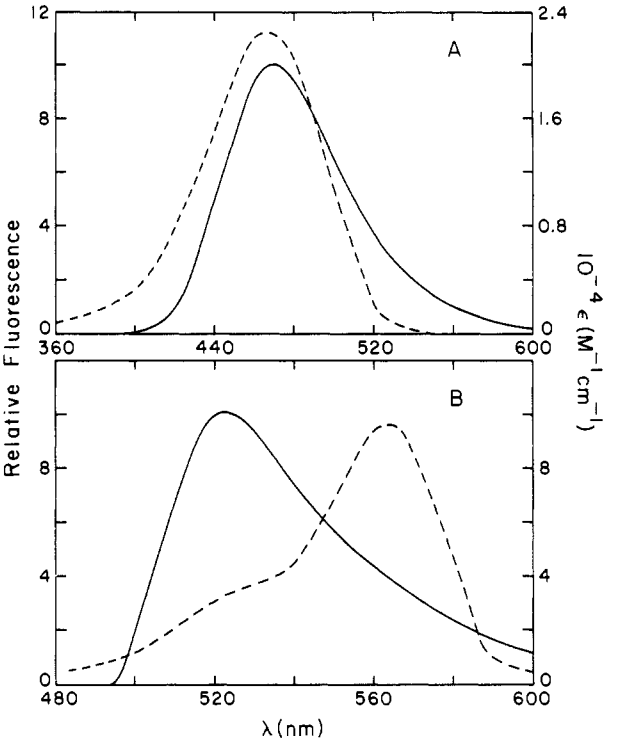

FIGURE 3: (A) Spectral overlap of the corrected fluorescence emission (387-nm excitation) for CPM (—), covalently bound to CF_1 at the γ-disulfide site, with the extinction coefficient (ε) of NBD-PE (---) in asolectin vesicles. (B) Spectral overlap of the corrected fluorescence emission (495-nm excitation) for fluoresceinylmaleimide (—), covalently bound to CF_1 at the γ-disulfide site, with the extinction coefficient of octadecylrhodamine (---) in asolectin vesicles. All spectra were taken in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA, at 25 °C.

included in Figure 1. Heat activation and modification of the γ-disulfide with fluoresceinylmaleimide resulted in no changes in the intrinsic fluorescent properties of the CPM label. The fluoresceinylmaleimide probe, on the other hand, exhibited some increase in freedom of rotation on the light-labeled CF_1 (polarization = 0.19) with respect to the enzyme containing no CPM label at this γ-sulfhydryl site (polarization = 0.24). The efficiency of energy transfer between the CPM adduct at the buried sulfhydryl and fluoresceinylmaleimide at the disulfide site was found to be 0.60 with a disulfide labeling stoichiometry of ≈ 1 mol of probe/mol of CF_1. If modification of the two reduced sulfhydryls formed after the disulfide reduction is severely restricted so that only a single sulfhydryl is labeled per enzyme, the distance between the sulfhydryl and disulfide sites is 47 Å. If the labeling of the reduced disulfide is random, the distance is 42 Å. (In this case, 50% of the disulfides would have one label, 25% two labels, and 25% no label.)

DSA γ-Disulfide as a Donor Site. To investigate the spatial relationships of the γ-sulfhydryls and nucleotide binding sites of CF_1 with respect to the membrane bilayer in the DSA complex, the γ-disulfide site on DSA was labeled with the fluorescent maleimide derivatives CPM and fluoresceinylmaleimide. The modified DSA was then reconstituted into asolectin vesicles containing acceptor fluorophores randomly distributed on the membrane surface. The distance of closest approach between the donor maleimide and the membrane surface can be determined by measuring the extent of energy transfer as a function of the surface density of acceptor molecules. The energy acceptors used were NBD-PE and octadecylrhodamine, and the energy donors were CPM and fluoresceinylmaleimide derivatives, respectively. The

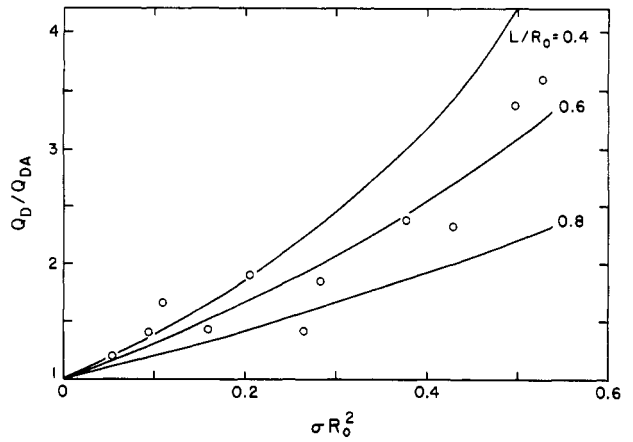

FIGURE 4: Plot of the ratio of the donor quantum yield in the absence and presence of acceptor (octadecylrhodamine), Q_D/Q_{DA}, vs. the normalized acceptor concentration, σR_0^2, for reconstituted DSA with fluoresceinylmaleimide covalently bound to the γ-disulfide site (σ is the surface density of the energy acceptor and R_0 was calculated from eq 5). The fluorescence measurements (495-nm excitation, 525-nm emission) were made in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA at 25 °C. The curves represent the theoretical quenching expected for the given values of L/R_0 calculated through a method of Monte Carlo simulation as described in the text.

fluorescence properties of labeled DSA were assumed to be the same as those for labeled CF_1. Overlap integrals were calculated from the spectra in Figure 3.

The results for the fluoresceinylmaleimide–octadecylrhodamine energy transfer system are presented in Figure 4 as a plot of the ratio of donor quantum yield in the absence and presence of acceptors, Q_D/Q_{DA} vs. the normalized acceptor concentration, σR_0^2. High background levels from vesicle scattering and fluorescent impurities in the crude asolectin mixture and the low protein/lipid ratios in the reconstituted system resulted in some scattering in the observed quenching ratios. The curves in this figure represent the theoretical quenching expected for the given values of L/R_0. (L is the distance of closest approach between donor and acceptor molecules.) A best fit of $L/R_0 = 0.59$ was obtained (see Materials and Methods), which corresponds to a distance, L, of 31 Å separating the γ-disulfide of DSA from the membrane surface. Unfortunately, accurate energy transfer measurements for the CPM–NBD-PE donor–acceptor pair could not be made due to the close overlap of the CPM and asolectin impurity emission fluorescences.

Discussion

In this study, fluorescence resonance energy transfer techniques have been used to investigate the spatial relationship between the nucleotide binding sites and specific sulfhydryl groups on the γ-subunit of CF_1, between sulfhydryl groups on the γ-subunit, and between sulfhydryl groups on the γ-subunit and the surface of the membrane in the reconstituted DSA system. The results are summarized in Table II as the calculated distances separating the energy donors and acceptors. For completeness, the distance between the light-labeled γ-sulfhydryl and the membrane surface is included.

The energy transfer determinations for CF_1 contain the implicit assumption that all donor quenching arises solely from energy transfer to the acceptor species and not from small environmental changes that may be induced by binding of the acceptor molecule to the enzyme complex. In support of this assumption, no changes are observed in the steady-state polarization of the donor or in the shape of its fluorescence emission spectrum (after correction for inner filter effects) upon binding of any of the acceptor compounds. Furthermore,

Table II: Summary of Distance Measurements (in Angstroms) for CF_1 and DSA[a]

	N_1	N_2	N_3	di-SH[b]	membrane surface[c]
L	52	51	51	42–47	45[d]
di-SH	>74	>74	62		31

[a] Separation distances between sites where di-SH represents the two γ-sulfhydryls exposed during heat activation of CF_1 or dithiothreitol treatment of DSA, L is the γ-sulfhydryl labeled in the light on thylakoids, and N1–N3 are nucleotide sites 1–3 on CF_1. [b] Limiting values for completely random and completely nonrandom labeling of di-SH, assuming L is equidistant from both sulfhydryls. [c] Distance of closest approach between labeled γ-sulfhydryls on reconstituted DSA and acceptor molecules on the membrane surface. [d] From Cerione et al. (1983); corrected for inner filter effects.

identical distances between the donor-labeled γ-sulfhydryls and specific nucleotide sites containing bound TNP-ATP are obtained independent of the presence or absence of bound nucleotide or nucleotide analogue at alternate binding sites. A discussion of the analysis of quenching profiles for the reconstituted DSA system has been presented elsewhere (Cerione et al., 1983). Although the difficulties in the experimental measurements cause some scatter in the data, the distance of closest approach is probably reliable to about ±15%.

Intramolecular distances determined through energy transfer techniques are dependent on the orientational freedom of the donor and acceptor species (Dale & Eisinger, 1974). In this work, the values of R_0 were calculated by assuming $\kappa^2 = 2/3$, the case in which donor and acceptor rotate freely and rapidly with respect to the fluorescence lifetime of the donor. The steady-state polarizations for the CPM and fluoresceinylmaleimide adducts on the γ-subunit of CF_1 can be used to calculate a *maximum* uncertainty of ±25–35% in the determination of R_0 and hence in the measured distances (Dale et al., 1979). However, a more realistic uncertainty of ±10–15% can be calculated with the inclusion of a moderate degree of acceptor rotational freedom and transfer depolarization.

Previous studies have suggested that the γ-subunit of CF_1 may play an important role in the regulation of catalytic activity for both the solubilized CF_1 and intact DSA complexes (Weiss & McCarty, 1977; Ketcham et al., 1984). The measured distances between the γ-sulfhydryl and nucleotide binding sites of CF_1 obtained in this study provide evidence that the nucleotide sites are quite far from the sulfhydryl groups on the γ-polypeptide (>50 Å). The pronounced effects of γ-sulfhydryl modification on ATP synthesis and hydrolysis (McCarty & Fagan, 1973; Nalin & McCarty, 1984; Moroney et al., 1984) must, therefore, be largely due to conformational changes. The spectroscopic and labeling characteristics of the light-labeled γ-sulfhydryl found in this and other studies (Nalin et al., 1983; Cerione et al., 1983) indicate that this cysteinyl residue is centrally located within the extrinsic enzyme complex. With this site as a reference point and from the known diameter of CF_1 (approximately 100 Å; Howell & Moudrianakis, 1967), the results presented in Table II suggest that all three nucleotide sites and the reduced γ-disulfide are located near the surface of the enzyme molecule. This structural characteristic would allow ready access to the external medium for possible involvement in either regulation or catalysis. Indeed, for the case of the disulfide site, previous studies have suggested that reduction and oxidation of CF_1 in vivo may be an important form of regulation (Mills et al., 1980; Mills & Mitchell, 1982). The oxidized γ-disulfide in isolated CF_1 is probably somewhat buried since reduction requires long exposure to high concentrations of dithiothreitol.

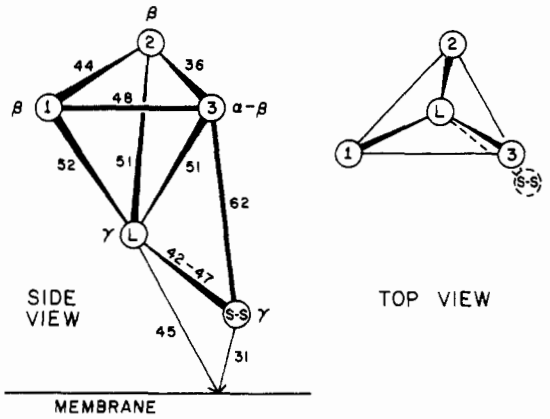

FIGURE 5: Diagrammatic illustration of the spatial relationships between the nucleotide binding sites, the γ-sulfhydryls, and the membrane surface of the reconstituted DSA system suggested from fluorescence resonance energy transfer measurements. L is the γ-sulfhydryl labeled in the light, S-S is the γ-disulfide, and the nucleotide binding sites are labeled 1–3. Views perpendicular and parallel to the bilayer surface are shown with distances in angstroms.

For the reconstituted DSA system, the γ-disulfide site was found to be relatively close to the vesicle surface (≈30 Å). If the structure of CF_1 is not appreciably altered when solubilized, this result suggests that the CF_1 complex on intact DSA is oriented so that the nucleotide binding sites are well removed from the membrane surface. A working model for the structural characteristics of DSA can be created with this assumption. A diagrammatic illustration of this model is presented in Figure 5. Structural maps of the nucleotide binding sites, γ-sulfhydryls, and the membrane bilayer are shown as viewed parallel and perpendicular to the bilayer surface. The spatial relationships between the nucleotide sites are also included (Cerione & Hammes, 1982). In this model, the nucleotide sites form a slightly skewed triangle near the top of CF_1. The two sulfhydryl sites, on the other hand, are located closer to the membrane with the light site in the interior and the disulfide near the surface of the enzyme complex. While the measured distances do not provide the necessary information to yield a unique orientation with respect to the membrane surface, the relative positions of each site are fixed due to the geometric constraints imposed by the interlocking spatial coordination. For example, the large distances between nucleotide sites 1 and 2 and the γ-disulfide (>74 Å) require that this sulfhydryl site be located directly below site 3. This asymmetry with respect to the γ-disulfide provides evidence that the nucleotide sites are not structurally equivalent. The relationship of this structural nonequivalence to the function of the enzyme remains to be elucidated.

The spatial orientation of the fourth cysteinyl residue of the γ-subunit remains to be determined although it is known to be close to the light-labeled sulfhydryl group (Weiss & McCarty, 1977; Cerione et al., 1983). The spatial relationships between the γ-disulfide and nucleotide binding sites of CF_1 and the essential tyrosines of the β-polypeptide have also been well characterized (Cantley & Hammes, 1976; Cerione & Hammes, 1982). Construction of a three-dimensional model shows that all of the distances measured thus far are self-consistent and predicts that the essential tyrosines are close to the light-labeled γ-sulfhydryl. Future energy transfer studies will attempt to verify these predictions and to extend this model to gain a more complete understanding of the structural characteristics of the coupling factor complex.

Acknowledgments

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References

Anderson, V. E., & Hammes, G. G. (1983) *Biochemistry 22*, 2995–3001.
Angelides, K. J., & Hammes, G. G. (1979) *Biochemistry 18*, 1223–1229.
Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.
Betcher-Lange, S. L., & Lehrer, S. S. (1978) *J. Biol. Chem. 253*, 3757–3760.
Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.
Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.
Cantley, L. C., & Hammes, G. G. (1975) *Biochemistry 14*, 2968–2975.
Cantley, L. C., & Hammes, G. G. (1976) *Biochemistry 15*, 9–14.
Cerione, R. A., & Hammes, G. G. (1981) *Biochemistry 20*, 3359–3365.
Cerione, R. A., Hammes, G. G. (1982) *Biochemistry 21*, 745–752.
Cerione, R. A., McCarty, R. E., & Hammes, G. G. (1983) *Biochemistry 22*, 769–776.
Dale, R. E., & Eisinger, J. (1974) *Biopolymers 13*, 1573–1605.
Dale, R. E., Eisinger, J. & Blumberg, W. E. (1979) *Biophys. J. 26*, 161–194.
Dewey, T. G., & Hammes, G. G. (1981) *J. Biol. Chem. 256*, 8941–8946.
Farron, F., & Racker, E. (1970) *Biochemistry 9*, 3829–3836.
Förster, Th. (1959) *Discuss. Faraday Soc. 27*, 7–17.
Holowka, D. A., & Hammes, G. G. (1977) *Biochemistry 16*, 5538–5545.
Howell, S. H., & Moudrianakis, E. N. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1261–1268.
Huang, C., & Mason, J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308–310.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.
Ketcham, S. R., Davenport, J. W., Warncke, K., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7286–7293.
Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 547–555.
Matsumoto, S., & Hammes, G. G. (1975) *Biochemistry 14*, 214–224.
McCarty, R. E., & Fagan, J. (1973) *Biochemistry 12*, 1503–1507.
Mills, J. D., & Mitchell, P. (1982) *Biochim. Biophys. Acta 679*, 75–83.
Mills, J. D., & Mitchell, P., & Schurmann, P. (1980) *FEBS Lett. 112*, 173–177.
Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58–62.
Moroney, J. V., Fullmer, C. S., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7281–7285.
Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56–60.
Nalin, C. M., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7275–7280.
Nalin, C. M., Bêliveau, R., & McCarty, R. E. (1983) *J. Biol. Chem. 258*, 3376–3381.
Parker, C. A., & Reese, W. T. (1966) *Analyst (London) 85*, 587–600.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Pick, U., & Racker, E. (1979) *J. Biol. Chem. 254*, 2793–2799.

Scott, T. G., Spencer, R. D., Leonard, M. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687–695.
Sippel, T. O. (1981) *J. Histochem. Cytochem. 29*, 1377–1381.
Snyder, B., & Freire, E. (1982) *Biophys. J. 40*, 137–148.
Takabe, T., & Hammes, G. G. (1981) *Biochemistry 20*, 6859–6864.
Weber, G., & Teale, F. W. J. (1957) *Trans. Faraday Soc. 53*, 646–655.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weiss, M. A., & McCarty, R. E. (1977) *J. Biol. Chem. 252*, 8007–8012.
Winget, G. G., Kanner, N., & Racker, E. (1977) *Biochim. Biophys. Acta 460*, 491–499.

Porcine Pancreatic α-Amylase Hydrolysis of Hydroxyethylated Amylose and Specificity of Subsite Binding†

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ABSTRACT: Hydrolysis of partially hydroxyethylated amylose by porcine pancreatic α-amylase gives rise to a number of hydroxyethylated di-, tri-, and tetrasaccharides, as well as larger products. No modified monosaccharides were detected. The structures of the products containing two to four D-glucose residues have been analyzed by chromatographic and enzymatic techniques. In no instance were these oligosaccharides modified in the reducing-end residue. The location of hydroxyethylated glucose residues within the oligosaccharides has been interpreted in terms of the ability of that (hydroxyethyl)glucose to bind productively at each of the five subsites of the enzyme active site. Results indicate that subsite 3, the subsite at which catalytic attack occurs, is especially sensitive to changes in the substrate and that unmodified glucose is required for productive binding at this subsite. Other subsites specifically allow binding of some (hydroxyethyl)glucose isomers, but not others. Hydroxyethylation is permitted at C-2, C-3, and C-6 for residues bound at subsite 1 and is permitted at C-6 and possibly at C-2 and C-3 for residues bound at subsite 5. However, substitution is permitted only at C-3 and C-6 for binding at subsite 2 and at C-2 and C-3 for binding at subsite 4.

Modified polysaccharides have previously been used to study the specificity and binding requirements for porcine pancreatic α-amylase (PPA)[1] and related enzymes. Several factors, however, have limited the use of this experimental approach. Naturally occurring polysaccharides have proved useful (Weill & Bratt, 1967; Saier & Ballou, 1968; Kainuma & French, 1969, 1970; Misaki et al., 1982; Takeda et al., 1983), but substrates containing desired structural features often are not available. Limitations in suitable synthetic procedures have also confined chemical approaches largely to polysaccharides modified at primary positions (Bines & Whelan, 1960; Weill et al., 1975), with a few exceptions (Kainuma & French, 1982). Moreover, analysis of enzymolysis products usually has been limited to trisaccharides or smaller products.

In this report, we describe the hydrolysis of hydroxyethylated amylose by PPA. Hydrolysis products included several modified oligosaccharides which were analyzed by various chemical and enzymatic techniques. Because this substrate contains hydroxyethyl substituents on C-2, C-3, and C-6 hydroxyls, it was possible to examine enzyme–substrate interactions throughout the PPA active site. Although the hydroxyethyl group may be expected to influence enzyme–substrate interactions through steric effects, these effects may be less pronounced than those observed for branched substrates. The hydroxyethyl group retains the hydroxyl moiety, which may help temper disruption of electronic interactions. In addition, the relatively small size of the PPA active site, 5 subsites compared to as many as 9–10 subsites for *Bacillus subtilis* α-amylase (Robyt & French, 1963; Thoma et al., 1971), allows a more complete interpretation of how substrate modifications affect binding at individual subsites.

Materials and Methods

Crystalline PPA (Worthington Biochemicals Corp.) and glucoamylase (*Rhizopus niveus* glucoamylase from Miles Laboratories and *Aspergillus niger* glucoamylase from Takamine Laboratory) were used without further purification. Specificity of both glucoamylases was identical for our uses. Superlose 500, a hydroxyethylated amylose with a degree of substitution of 0.1–0.2 (i.e., 1–2 substituents per 10 glucose residues), was obtained from Stein-Hall Co. Complete acid hydrolysis of this material, followed by gas chromatography, indicated that approximately 50% of the hydroxyethyl groups were located at C-2, 30% at C-6, and 20% at C-3.

Evaporations were conducted below 40 °C under reduced pressure. Total carbohydrate content and reducing sugar content were analyzed by using the orcinol–sulfuric acid and alkaline ferricyanide methods, respectively, as adapted for use

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[1] Abbreviations: PPA, porcine pancreatic α-amylase; Me_3Si, trimethylsilyl; ds, degree of substitution; HE, hydroxyethyl ($HOCH_2CH_2$). Hydroxyethylated oligosaccharides are named by first indicating which hydroxyl group is modified and then by indicating with a superscript numeral which glucose residue holds that group. Residues are numbered from the reducing end. Hence, 3^2-HE-maltotriose is maltotriose containing a hydroxyethyl group on the oxygen at C-3 of the second residue.

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