Characterization of Sulfhydryl Groups on Chloroplast Coupling Factor 1 Exposed by Heat Activation†

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ABSTRACT: Two sulfhydryl groups in the γ subunit of solubilized chloroplast coupling factor 1 (CF_1) are exposed by heat activating the enzyme. These two groups have been selectively labeled with [^{3}H]-*N*-ethylmaleimide, *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide (NBPM), and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM). Modifying these groups did not appreciably affect Ca^{2+}-ATPase activity, the ability of the enzyme to bind quercetin and 1,N^6-ethenoadenosine diphosphate (ϵADP), or the ability of the enzyme to react with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Fluorescence resonance energy transfer was used to measure the distance from the sulfhydryl groups on the γ subunit to the quercetin sites, the NBD-Cl reactive sites, and the tight nucleotide sites on CF_1 using the donor-acceptor pairs NBPM-quercetin, NBPM-NBD, and ϵADP-DDPM, respectively. The distance from the sulfhydryl groups to the quercetin sites was found to be less than 30 Å, the distance to the NBD-Cl reactive sites was 34–41 Å, and the distance to the tight nucleotide sites was greater than 40 Å. A three-dimensional symmetrical model is proposed for the relative positions of sites on CF_1.

The purified coupling factor 1 (CF_1)[1] from spinach chloroplasts contains five different subunits (α, β, γ, δ, and ϵ) with molecular weights of 59000, 56000, 37000, 17500, and 13000 (Lien et al., 1972). The reactivity of sulfhydryl groups has been investigated on both the membrane bound and the solubilized enzyme. Farron and Racker (1970) reported two sulfhydryl groups reacted with iodoacetamide on the latent solubilized enzyme and two additional groups were exposed by heat activation. Deters et al. (1975) observed an increase in incorporation of iodoacetamide into the γ subunit of CF_1 upon heat activation. McCarty and Fagan (1973) reported incorporation of NEM into the γ and ϵ subunits of CF_1 bound to chloroplasts in the dark and additional incorporation into the γ subunit upon exposure to light. Amino acid analysis of the purified subunits indicates 1.8, 3.1, 5.8, and 1 half-cystine per subunit for the α, β, γ, and ϵ subunits, respectively (Nelson et al., 1972, 1973), and the solubilized enzyme has 8 cysteines and 2 cystines per mole (Farron and Racker, 1970).

In this work, a procedure is described for reacting NEM specifically with 2 sulfhydryl groups on the γ subunit of heat-activated CF_1. The NEM analogues, NBPM and DDPM, also react at these sites, and the modified enzyme retains Ca^{2+}-ATPase activity. Fluorescence resonance energy transfer is used to measure the distances between the sulfhydryl groups on the γ subunit and the quercetin binding sites (Cantley and Hammes, 1976), the tight nucleotide binding sites, and the NBD-Cl reactive sites (Cantley and Hammes, 1975a,b) with the donor-acceptor pairs NBPM-quercetin, ϵADP-DDPM, and NBPM-NBD-Tyr. A symmetrical model is proposed to summarize the measured distances between sites on CF_1.

Experimental Section

Materials. The [^{3}H]ϵADP was prepared as previously described (Cantley and Hammes, 1975a). The [^{3}H]NEM was purchased from New England Nuclear, the NBD-Cl from Pierce Chemical Co., the sodium dodecyl sulfate from Bio-Rad Laboratories, and the NCS Tissue Solubilizer from Amersham Searle. The quercetin and NBPM were obtained from Eastman Chemical Co., and the quinine sulfate and DDPM were purchased from Aldrich Chemical Co. The NBPM-Cys was synthesized according to the procedure of Kanaoka et al. (1967). All other chemicals were the best available commercial grades, and all solutions were prepared with deionized distilled water.

CF_1 Preparation. The CF_1 was prepared by known procedures (Lien and Racker, 1971). An extinction coefficient of 0.476 ml/(mg cm) (Cantley and Hammes, 1975a) and a molecular weight of 325000 (Farron, 1970) were used to determine molar concentrations of CF_1. The heat-activated enzyme (Lien and Racker, 1971) had a specific ATPase activity of 10–12 μmol/(mg min) using the pH stat technique with the following assay conditions: 5 m*M* ATP, 5 m*M* $CaCl_2$, ca. 6 μg/ml of CF_1 (pH 8.0), 23°.

Preparation of [^{3}H]NEM-CF_1, NBPM-CF_1, DDPM-CF_1, and NBPM-NBD-CF_1. The ammonium sulfate precipitate of CF_1 was dissolved in a small volume of 40 m*M* Tris-Cl and 2 m*M* EDTA (pH 7.0) and eluted through a Sephadex G-25 (medium) column (0.6 cm i.d. × 20 cm) equilibrated with the same buffer. The effluent was monitored at 280 nm by passing it through a microflow cell (Helma Cell, Inc., Type 178-QS, 10-mm path length) in a Cary 14 recording spectrophotometer. Unlabeled NEM was added to the protein fraction to a final concentration of 10

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[1] Abbreviations used are: CF_1, chloroplast coupling factor 1; NEM, *N*-ethylmaleimide; NBPM, *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; ϵADP, 1,N^6-ethenoadenosine diphosphate; NBPM-Cys, *S*-[*N*-(*p*-(2-benzoxazolyl)phenyl)succinimido]cysteine; [^{3}H]NEM-CF_1, [^{3}H]NEM reacted specifically with two cysteine groups in the γ subunit of CF_1; NBPM-CF_1, NBPM reacted specifically with two cysteine groups in the γ subunit of CF_1; DDPM-CF_1, DDPM reacted specifically with two cysteine groups on the γ subunit of CF_1; NBPM-NBD-CF_1, two cysteine groups on the γ subunit of CF_1 reacted with NBPM and 1 or 2 tyrosine groups on the β subunit reacted with NBD-Cl; F_1, mitochondrial coupling factor 1.

mM. After 1 hr at room temperature, the protein was precipitated with ammonium sulfate and centrifuged at 18000g, 4° for 10 min. The precipitate was redissolved in 40 mM Tris-Cl and 2 mM EDTA (pH 8.0) and eluted through a Sephadex G-50 column (1 cm i.d. × 25 cm) equilibrated with the same buffer. The protein fraction was collected and heat activated by the procedure of Lien and Racker (1971). The enzyme was then precipitated, centrifuged, and eluted through the Sephadex G-25 column (pH 7.0) as before. The protein fraction was reacted with [^{3}H]NEM (230 Ci/mol; 16 μM [^{3}H]NEM-4 μM CF_1 for 3 hr), NBPM (200 μM NBPM-5-20 μM CF_1 for 30 min), or DDPM (150 μM DDPM-30 μM CF_1 for 1 hr) to form [^{3}H]NEM-CF_1, NBPM-CF_1, or DDPM-CF_1, respectively. The protein was precipitated, centrifuged, and eluted through the Sephadex G-25 column (pH 7.0) as above to remove any unreacted reagent. The stoichiometry of the [^{3}H]NEM-CF_1 was 2.05 mol of [^{3}H]NEM/mol of CF_1 as determined by the specific radioactivity. The stoichiometry of the NBPM-CF_1 was 1.95 mol of NBPM/mol of CF_1 using an extinction coefficient, ϵ, of 3.24×10^4 M^{-1} cm^{-1} at 308 nm as found for the model compound S-[N-(p-(2-benzoxazolyl)phenyl)succinimido]cysteine ethyl ester hydrochloride (Kanaoka et al., 1967) and correcting for the protein absorbance at 308 nm (ϵ 0.048 ml/(mg cm)) and the NBPM absorbance at 280 nm (ϵ 1.9×10^4 M^{-1} cm^{-1}). The stoichiometry of DDPM-CF_1 was 1.84 mol of DDPM/mol of CF_1 using an extinction coefficient of 3.0×10^3 M^{-1} cm^{-1} at 440 nm as found for the model compound DDPM-N-acetylcysteine (Gold and Segal, 1964) and correcting for protein absorbance at 440 nm (ϵ 0.003 ml/(mg cm)) and DDPM absorbance at 280 nm (ϵ 1.53×10^4 M^{-1} cm^{-1}). Since the extinction coefficient of DDPM at 440 nm is small, the stoichiometry of DDPM-CF_1 was checked by reacting the modified enzyme (7 μM) with 40 μM [^{3}H]NEM for 1 hr and removing the excess [^{3}H]NEM by gel filtration. Less than 0.02 mol of [^{3}H]HEM/mol of CF_1 was incorporated indicating that 2 mol of cysteine/mol of CF_1 was blocked by DDPM.

The NBPM-NBD-CF_1 was prepared by reacting NBPM-CF_1 (10 μM) with NBD-Cl (400 μM) for various time periods (15 min-4 hr) in 40 mM Tris-Cl and 2 mM EDTA (pH 7.0) at 23°. The excess NBD-Cl was removed by eluting the enzyme through the Sephadex G-25 column (pH 7.0) as described above. The stoichiometry of the NBPM-NBD-CF_1 varied from 0.48 to 2.0 mol of NBD/mol of CF_1 as determined by the extinction coefficient of 1.07×10^4 M^{-1} cm^{-1} at 400 nm for NBD reacted with a tyrosine group on CF_1 (Cantley and Hammes, 1975a).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The general method of Weber and Osborn (1969) was used with the specific procedure described by Lad and Hammes (1974). The 10% half-cross-linked gels were prepared by adding 1 ml of 0.15% ammonium persulfate, 30 μl of redistilled tetramethylenediamine, and 10 ml of 0.2 M sodium phosphate, 0.2% sodium dodecyl sulfate (pH 7.0) to 9 ml of acrylamide solution (22.2 g of acrylamide and 0.3 g of N,N-methylenebisacrylamide in 100 ml of water). After electrophoresis of the [^{3}H]NEM-CF_1, the stained gels were frozen with dry ice and cut into 1-2-mm sections with a razor blade, noting the sections containing protein. The sections were incubated in 1 ml of NCS solubilizer (9:1, NCS-water) in sealed scintillation vials for 3 hr at 50°. After cooling, 10 ml of scintillation fluid (0.2 g of 2,5-diphenyloxazole and 5.0 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. of toluene) was added and the radioactivity was assayed in a Bechman LS-255 liquid scintillation counter.

Difference Spectra Measurements. The difference spectrum titration of quercetin binding to NBPM-CF_1 was performed as described previously (Cantley and Hammes, 1976) in 40 mM Tris-Cl, 2 mM EDTA, and 1 mM dithiothreitol (pH 7.0).

Steady State Fluorescence Measurements. A Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer was used for all steady state fluorescence measurements. The quantum yield of NBPM bound to CF_1 was measured by a comparative method (Parker and Rees, 1966) using quinine sulfate in 0.1 N H_2SO_4 as a standard and correcting for the polarized emission of NBPM (Shinitzky, 1972) as previously described (Cantley and Hammes, 1976).

The quenching of ϵADP fluorescence (320-nm excitation, 400-nm emission) by DDPM was measured by adding 3-5 μl of DDPM (10 mM in dimethyl sulfoxide) to 200 μl of 30-90 μM CF_1 (treated with 10 mM NEM then heat activated as described above) preequilibrated with 50-140 μM [^{3}H]ϵADP for 2 hr in 0.1 M NaCl, 50 mM Tris-Cl, and 2 mM $MgCl_2$ (pH 7.0) in a fluorescence microcell (0.3 cm × 0.3 cm). The fluorescence was measured periodically for 1 hr and the free and total nucleotide concentrations were then immediately determined (in about 5 min) using the forced dialysis technique (Cantley and Hammes, 1973). As a control experiment, the CF_1 was equilibrated with unlabeled ADP (800 μM) before adding [^{3}H]ϵADP. The DDPM was then added and the fluorescence monitored as above. The ADP prevented [^{3}H]ϵADP binding so that the inner filter effect of DDPM reacting with the enzyme could be determined.

The quenching of NBPM-CF_1 fluorescence (312-nm excitation, 366-nm emission) by quercetin was measured by titrating 2.9 μM NBPM-CF_1 (2.0 mol/mol, NBPM/CF_1) in 40 mM Tris-Cl and 2 mM EDTA (pH 7.0) with 1.0 mM quercetin in 95% ethanol while monitoring the NBPM fluorescence. In the control experiment, NBPM-Cys in the same buffer was titrated with quercetin and the fluorescence monitored using the same instrument settings, in order to correct for inner filter absorbance of quercetin. The fluorescence microcells were used to minimize the absorbance.

The efficiency of energy transfer from NBPM to NBD-Tyr on NBPM-NBD-CF_1 was measured by adding 1 μl of β-mercaptoethanol to 200 μl of ~5 μM NBPM-NBD-CF_1 in the fluorescence microcell and monitoring the increase in fluorescence (312-nm excitation, 366-nm emission) with time as the NBD-Tyr was reduced (approximately 2.5 hr).

All fluorescence measurements were corrected for light scattering and inner filter or dilution effects. The fluorescence polarization measurements were corrected for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Azumi and McGlynn, 1962).

Fluorescence Lifetime Measurements. The fluorescence lifetime of NBPM-CF_1 (312-nm excitation, 370-nm emission) was measured with an Ortec Model 9200 nanosecond fluorescence spectrophotometer as previously described (Cantley and Hammes, 1976).

Results

Figure 1 is a representation of the migration of subunits of [^{3}H]NEM-CF_1 in sodium dodecyl sulfate polyacrylam-

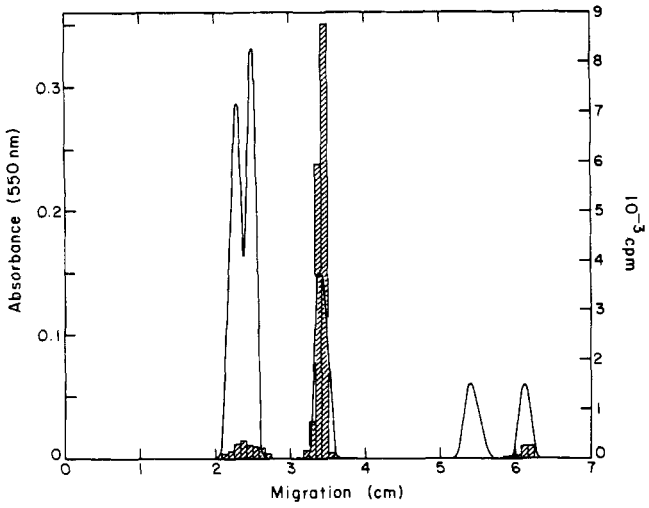

FIGURE 1: The migration of [^{3}H]NEM-CF_1 (2.05 mol/mol; [^{3}H]NEM/CF_1) subunits in sodium dodecyl sulfate polyacrylamide gel electrophoresis. The solid lines represent the absorbance of stained gels at 550 nm, and the hatched bars represent the tritium counts per minute in gel slices. The [^{3}H]NEM-CF_1 preparation and procedure are described in the Experimental Section.

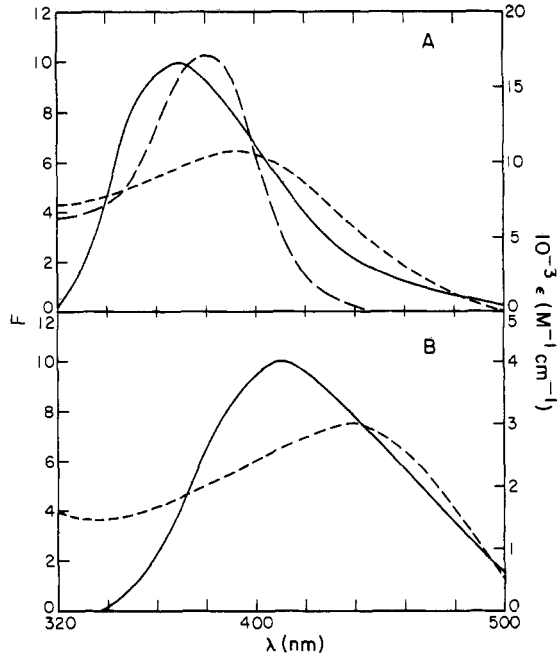

FIGURE 2: (A) Overlap of the corrected fluorescence emission (F) of NBPM reacted with CF_1 excited at 312 nm (—) with the extinction coefficient of (ϵ) of quercetin (- -) bound to CF_1 and the difference extinction coefficient (ϵ) of NBD-Tyr (- - - -). (B) Overlap of the corrected fluorescence emission (F) of ϵADP excited at 320 nm (—) with the extinction coefficient (ϵ) of DDPM-N-acetylcysteine (- - - -). The DDPM-N-acetylcysteine spectrum was measured by Gold and Segal (1964) in 0.1 M sodium phosphate (pH 7.0). All other spectra were measured in 40 mM Tris-Cl and 2 mM EDTA (pH 7.0); 1 mM dithiothreitol was present when measuring the quercetin spectrum.

ide gel electrophoresis. The [^{3}H]NEM-CF_1 was prepared by reacting solubilized CF_1 with 10 mM NEM (unlabeled) for 1 hr, heat activating the enzyme, and then reacting with [^{3}H]NEM as described in the Experimental Section. The reaction saturated at 2 mol of [^{3}H]NEM/mol of CF_1 and 90% of the radioactivity was incorporated into the γ subunit. Reacting 10 mM NEM with latent CF_1 for 1 hr incorporates 1 mol of NEM into the γ subunit, 1 mol into the ϵ subunit, and approximately 0.3 mol into the β subunit per mol of CF_1.[2] The NEM analogues NBPM and DDPM also reacted with two sulfhydryl groups exposed by heat activation. These compounds were not available in radioactive form; however, it was possible to visualize a blue fluorescent band in unstained sodium dodecyl sulfate polyacrylamide gels of NBPM-CF_1 with an ultraviolet lamp. This single blue band migrated at the same rate as the γ subunit.

The specific activity of the enzyme was monitored after its reaction with 10 mM NEM, after its heat activation, and during its reaction with NBPM at pH 7.0. The specific activity after heat activation was 8.5 μmol/(mg min) (compared with 10 μmol/(mg min) for the enzyme not treated with NEM) and the activity was unchanged during the 30-min reaction with 200 μM NBPM. However, it was noted that the NEM treated, heat-activated enzyme is unstable at pH 8.0, and solutions of this enzyme become cloudy after a few minutes.

The fluorescence emission spectrum of NBPM reacted with CF_1 overlaps the absorption spectrum of quercetin (see Figure 2A) so that fluorescence resonance energy transfer may occur between the two probes if they are sufficiently close. It was first necessary to characterize the binding of quercetin to NBPM-CF_1. The quercetin binding was measured by a series of difference spectrum titrations in which both the quercetin and enzyme concentrations were varied. The solutions contained 40 mM Tris-Cl, 2 mM EDTA, and 1 mM dithiothreitol (pH 7.0), and the difference absorbance was monitored at 420 nm. The data were analyzed by a least-squares iterative technique using eq 1 which assumes two types of noninteracting sites with equal difference extinction coefficients (Cantley and Hammes, 1976). Here r

$$r = \frac{\Delta a}{\Delta\epsilon(E_0)} = \frac{n_1(L)}{K_1 + (L)} + \frac{n_2(L)}{K_2 + (L)} = r_1 + r_2$$

$$(L) = (L_0) - \Delta a/\Delta\epsilon \qquad (1)$$

is the number of moles of quercetin bound per mole of CF_1, the n_i are the number of sites of type i per mole of enzyme with intrinsic dissociation constants K_i, (E_0) is the total enzyme concentration, $\Delta\epsilon$ is the difference extinction coefficient at 420 nm, Δa is the measured difference in absorbance at 420 nm, (L_0) is the total quercetin concentration, and (L) is the concentration of unbound quercetin. Whole number values were assigned to n_1 and n_2; $\Delta\epsilon$, K_1, and K_2 were allowed to vary. Similar fits to the data were found for $n_1 = 1$ or $n_1 = 2$, and the corresponding parameters determined were $n_1 = 1$, $K_1 = 6.2$ μM, $n_2 = 14$, $K_2 = 0.5$ mM, $\Delta\epsilon = 1.36$ M^{-1} cm^{-1}, and $n_1 = 2$, $K_1 = 20.8$ μM, $n_2 = 9$, $K_2 = 0.94$ mM, $\Delta\epsilon = 1.57 \times 10^3$ M^{-1} cm^{-1}. Equation 1 is the simplest which adequately fits the data and higher values for n_1 gave poor fits of the data. The best fit of the quercetin binding to native CF_1 in 0.1 M NaCl, 50 mM Tris-Cl, 5 mM $CaCl_2$, and 1 mM dithiothreitol (pH 8.0) to eq 1 was obtained with the parameters $n_1 = 2$, $K_1 = 19.5$ μM, $n_2 = 2$, $K_2 = 94.5$ μM, and $\Delta\epsilon$ (at 410 nm) $= 4.43 \times 10^3$ M^{-1} cm^{-1} (Cantley and Hammes, 1976). Thus, both sets of data are consistent with two tight quercetin sites with intrinsic dissociation constants of $\sim$20 μM. The modified enzyme at pH 7.0 appears to have more nonspecific binding, possibly because quercetin is less ionized at this pH and exhibits more hydrophobic binding. The binding to the modified enzyme could not be studied at pH 8.0 since the enzyme is unstable at this pH. The extent of quercetin bind-

[2] Personal communication from Dr. Richard McCarty.

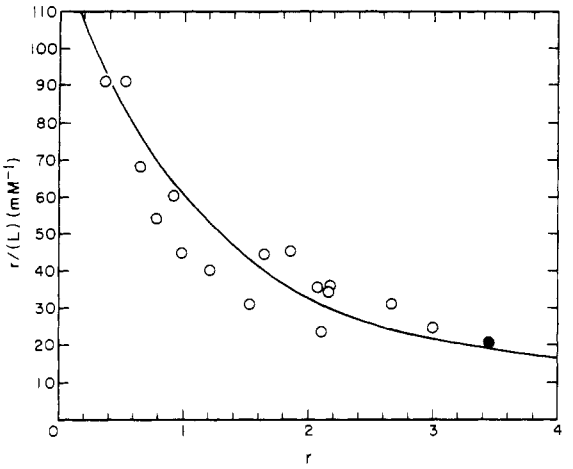

FIGURE 3: A plot of $r/(L)$ vs. r, where r is the number of moles of quercetin bound per mole of NBPM-CF_1 and (L) is the concentration of free quercetin. The data were obtained by difference spectrum titrations (○) 4.60–15.6 μM NBPM-CF_1 and 9.9–144.2 μM quercetin, and by forced dialysis (●) 9.67 μM NBPM-CF_1 and 190 μM quercetin. The solutions contained 40 mM Tris-Cl, 2 mM EDTA, and 1 mM dithiothreitol (pH 7.0), and the free ligand concentration in the difference spectrum titration was determined from an iterative least-squares fit of the data to eq 1.

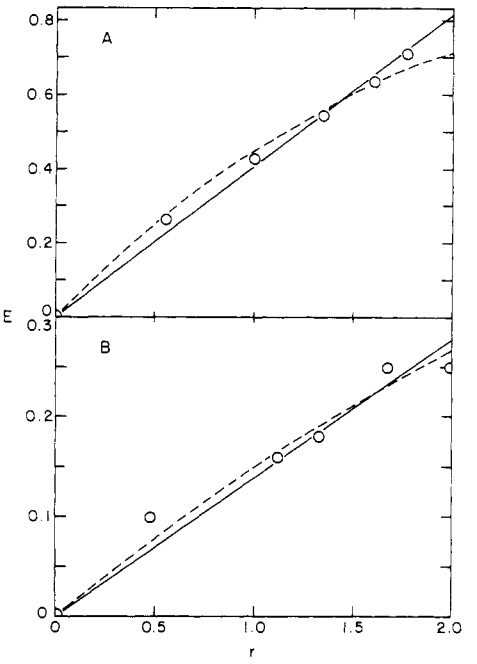

FIGURE 4: (A) A plot of the efficiency of energy transfer, E, from NBPM reacted with CF_1 to quercetin vs. the number of moles of quercetin bound to the "tight" sites per mole of CF_1, r. (B) The efficiency of energy transfer, E, from NBPM reacted with CF_1 to NBD-Cl reacted with a tyrosine group on CF_1 vs. the number of moles of NBD-Tyr per mole of CF_1, r. The solid lines represent linear extrapolations of the data to $r = 2$, and the dotted lines were calculated using eq A3 (Cantley and Hammes, 1976) and the parameters in Table I.

ing at high concentration was also estimated using the forced dialysis technique (Cantley and Hammes, 1973). The results of the spectral titrations and forced dialysis are presented in Figure 3 as a plot of $r/(L)$ vs. r. The curve has been calculated with the parameters given above for $n_1 = 2$. The scatter in the data is a reflection of the small difference extinction coefficient.

The efficiency of fluorescence resonance energy transfer, E, is given by (Förster, 1959, 1965)

$$E = 1 - \tau_{D\to A}/\tau_D = 1 - Q_{D\to A}/Q_D = 1 - F_{D\to A}/F_D \quad (2)$$

where $\tau_{D\to A}$, $Q_{D\to A}$, and $F_{D\to A}$ are the fluorescence lifetime, quantum yield, and relative fluorescence intensity of the donor in the presence of the acceptor, and τ_D, Q_D, and F_D are the fluorescence lifetime, quantum yield, and fluorescence intensity of the donor in the absence of transfer.[3] The efficiency of energy transfer from NBPM reacted with CF_1 (2.0 mol/mol, NBPM/CF_1) to quercetin was measured as described in the Experimental Section, using eq 2. The stoichiometry of quercetin bound to the tight and weak sites was determined from the total quercetin and enzyme concentrations using an iterative fit to eq 1 with the parameters $n_1 = 2$, $K_1 = 20.8$ μM, $n_2 = 9$, and $K_2 = 0.94$ mM. It is a reasonable assumption that the tight quercetin sites are responsible for most of the energy transfer since an efficiency of 0.42 is measured when r_1 is 1.0 and r_2 is less than 0.2. In Figure 4A, the efficiency of transfer from NBPM-CF_1 to quercetin (assuming equal quenching of both donors) is plotted vs. r_1. The efficiency linearly extrapolated to $r_1 = 2$ is 0.81. The quantum yield of NBPM reacted with CF_1 is 0.318 and the polarization is 0.299. Measurements of the polarization, P, of NBPM reacted with cysteine in various sucrose solutions were used to construct a plot of $1/P$ vs. T/η (Perrin, 1926) where η is the viscosity and T is the absolute temperature. Extrapolation to infinite viscosity gave a limiting polarization, P_0, of 0.351. The fluorescence lifetime of NBPM reacted with CF_1, 1.7 nsec, was too short to allow an accurate efficiency of energy transfer to be determined from the fluorescence lifetime.

Fluorescence energy transfer measurements were made between the NBPM reactive site and the NBD-Tyr site using the double modified enzyme NBPM-NBD-CF_1 as described in the Experimental Section. Although the second tyrosine group reacts with NBD-Cl much slower than the first on latent CF_1 (Cantley and Hammes, 1975a; Deters et al., 1975), both tyrosine groups react readily on the heat-activated enzyme. Figure 4B is a plot of the efficiency of energy transfer from NBPM reacted with CF_1 to NBD-Cl reacted with CF_1 vs. the number of moles of NBD-Cl reacted per mole of CF_1. A linear extrapolation of the data to 2 mol of NBD-Cl reacted gives an efficiency of 0.276.

As shown in Figure 2B, the N-acetylcysteine adduct of DDPM overlaps the fluorescence emission of ϵADP. Furthermore ϵADP has been shown to bind tightly to two sites on CF_1 (Cantley and Hammes, 1975a). These two probes were used to measure the distance from the tight nucleotide sites to the cysteine groups on the γ subunit exposed by heat activation. Binding measurements made by the forced dialysis technique (Cantley and Hammes, 1973) showed that reacting the latent enzyme with 10 mM NEM for 1 hr did not inhibit ADP binding to the two tight nucleotide sites. Measurements of the binding of [^{3}H]ϵADP to the NEM treated, heat-activated enzyme before and after reaction with DDPM showed that DDPM does not affect the ϵADP binding significantly. The quantum yield of ϵADP bound to CF_1 in the presence of DDPM was calculated as previously described for the determination of the ϵADP quantum yield in the presence of quercetin (Cantley and Hammes, 1976). The efficiency of transfer calculated using eq 2 was 0.04 ±

[3] Strictly speaking the measured fluorescence intensity should be corrected for the change in polarization of the donor emission in the presence and absence of the acceptor (Shinitzky, 1972). This correction is negligible in the systems under consideration here.

Table I: Energy Transfer Parameters.

Donor	Acceptor	R_0[a] (Å)	E[b]	R_1[c] (Å)	R_2[c] (Å)	R_3[c] (Å)	R_4[c] (Å)
NBPM	Quercetin	31.5	0.81[d]	24.9	0		
			0.71[e]			30.5	0
NBPM	NBD-Tyr	31.2	0.28[d]	36.7	30.2		
			0.27[e]			41.4	34.2
ϵADP	DDPM	27.7	0.04	47.0	41.6	52.8	46.7

[a] Assuming $K^2 = 2/3$. [b] Efficiency observed assuming equal quenching of both donors; the approximate uncertainty in the efficiencies is ±10% except for the last entry where the uncertainty is approximately ±0.04. [c] R_1–R_4 were calculated from eq A1–A4, respectively, in Appendix to the accompanying paper (Cantley and Hammes, 1976). [d] Linear extrapolation to two acceptors bound/enzyme molecule. [e] Extrapolation to two acceptors bound/enzyme molecule according to eq A3.

0.04. The stoichiometry of ϵADP bound was 0.46–0.56 mol/mol of CF_1 and 2 mol of DDPM was reacted per mol of CF_1.

Discussion

The results presented here clearly indicate that heat activating CF_1 makes two reactive sulfhydryl groups in the γ subunit accessible to NEM. By blocking the accessible sulfhydryl groups on latent CF_1 with NEM before heat activation, it was possible to selectively react [^{3}H]NEM or the fluorescence and absorption probes NBPM and DDPM with two cysteine groups on the γ subunit. The observed stoichiometry and location of these exposed groups are consistent with the results of Farron and Racker (1970), and Deters et al. (1975). These results along with the observed stimulation of ATPase activity by dithiothreitol in both solubilized and membrane bound CF_1 (Farron and Racker, 1970; McCarty and Racker, 1967) suggest that the activation process either exposes two previously buried cysteine groups in the γ subunit, or reduces a disulfide bond. McCarty and Fagan (1973) observed light stimulated incorporation of NEM into the γ subunit of chloroplast bound CF_1 with a resulting inhibitory effect on photophosphorylation suggesting these sulfhydryl groups play an important role in photophosphorylation. The excitation and reduction of a disulfide bond has been suggested to have a regulatory function in several transport systems (Czech et al., 1974; Zipursky et al., 1974; Morgan et al., 1965).

Reacting the two sulfhydryl groups on the γ subunit with the NEM analogues does not inhibit the Ca^{2+}-ATPase activity. The modification also did not prevent ϵADP binding, quercetin binding, or reaction of the enzyme with NBD-Cl. Thus, the exposed sulfhydryl groups are not at the ATP hydrolysis site, the tight nucleotide binding sites, or the quercetin binding sites. NBD-Cl has been shown to react with a tyrosine group in the β subunit of CF_1 and quercetin inhibits the Ca^{2+}-ATPase activity of enzyme containing only α and β subunits (Deters et al., 1975); however, the subunit location of the tight nucleotide sites is still unknown. In view of the inhibition of NEM incorporation into the γ subunit of the chloroplast bound enzyme by low concentrations of nucleotides (Magnusson and McCarty, 1975), the distance between the tight nucleotide sites and the NEM sites is of some interest.

The usual problems of environmental changes at the donor site due to acceptor binding, and the donor–acceptor dipole orientations (cf. Dale and Eisinger, 1974) must be considered in interpreting the energy transfer measurements. Although the quenching of NBPM–CF_1 fluorescence by the acceptors NBD-Tyr and quercetin may be attributed to a change in donor environment, the absence of any shift in the NBPM fluorescence spectrum makes this unlikely. In the case of energy transfer measurements between ϵADP and DDPM–CF_1, virtually no change was noted in the fluorescence emission spectrum of the donor ($E = 0.04 \pm 0.04$).

The critical transfer distance, R_0, characteristic of a given donor–acceptor pair (Förster, 1959) was calculated as previously described (Cantley and Hammes, 1976) using a value for the orientation factor, K^2, of ⅔ which assumes the donor and acceptor dipoles are rotating rapidly relative to the fluorescence lifetime of the donor. The calculated values of R_0 are presented in Table I. If only the donor is free to rotate, then K^2 may vary from ⅓ to 4/3 (Wu and Stryer, 1972) which allows R_0 to vary ±11% from the value calculated assuming $K^2 = ⅔$. A mean rotational correlation time, ϕ, may be calculated for the NBPM reacted with CF_1 from the fluorescence lifetime, τ, the measured polarization, P, and the polarization at infinite viscosity P_0, using eq 3 (Weber, 1952):

$$\phi = \rho/3 = \tau/[(1/P - ⅓)/(1/P_0 - ⅓) - 1] \qquad (3)$$

where ρ is the rotational relaxation time. Using the values $\tau = 1.7$ nsec, $P = 0.299$, and $P_0 = 0.351$, $\phi = 8.63$ nsec. This is much shorter than the rotational correlation time of the enzyme (~100 nsec, assuming a 100-Å sphere) indicating considerable freedom of the donor to rotate on the enzyme. The rotational correlation times of the acceptors quercetin and NBD-Tyr cannot be determined since they do not fluoresce. The donor ϵADP is somewhat rigid (Cantley and Hammes, 1975b); however the acceptor DDPM–CF_1 probably has a mobility similar to NBPM–CF_1. Thus the donor and acceptor dipoles very probably exist in a variety of different relative orientations. The existence of multiple donor and acceptor sites also makes the extreme values of K^2 (0 or 4) unlikely for all the possible donor–acceptor interactions.

All three energy transfer measurements involve two donor sites and two acceptor sites. The Appendix in the accompanying paper (Cantley and Hammes, 1976) presents the possible arrangements of two donors and two acceptors which allow calculations of the extreme values for the donor–acceptor distance, R, from the measured efficiencies and R_0. The dotted lines in Figures 4A and B represent least-squares fits of the data for NBPM–quercetin and NBPM–NBD-Tyr energy transfer to eq A3 of the Appendix which assumes both acceptor sites are equally quenching both donors. The solid lines represent linear extrapolations of the data assuming only one acceptor is involved in transfer from each donor. The uncertainty in the data does not allow distinction between these two models. For the donor–acceptor pair ϵADP–DDPM, the measured efficiency was within the experimental error of the technique

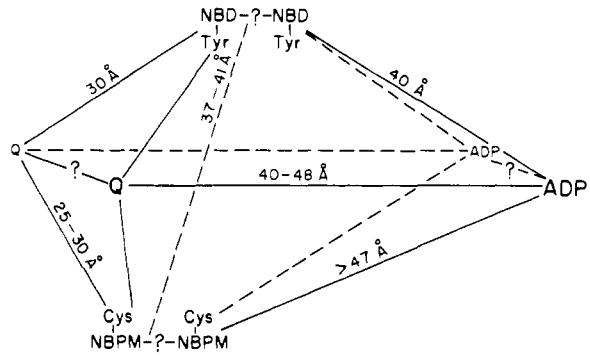

FIGURE 5: A three-dimensional schematic representation of the relative position of sites on solubilized CF_1 with an assumed symmetry. The NBD-Tyr sites are on the β subunit and are proposed to be at or near the active site for ATP hydrolysis. The NBPM sites are on the γ subunit. The subunit location of the quercetin sites (represented by a *Q*) and the ADP sites is not yet known.

(±4%) suggesting a large separation for this pair. The distances calculated using the models from the Appendix of the accompanying paper are presented in Table I.

Figure 5 presents a symmetrical structural model summarizing the distances calculated from fluorescence energy transfer measurements present in this and two preceding papers (Cantley and Hammes, 1975b, 1976). The NBD-Tyr sites are located on the β subunit of CF_1 (Deters et al., 1975) and are very likely near the active site for ATP hydrolysis on both F_1 and CF_1 (Deters et al., 1975; Ferguson et al., 1975a,b; Cantley and Hammes, 1975a). The distance between the two NBD-Tyr sites is uncertain although the negative cooperativity in reacting NBD-Cl with native CF_1 and the fact that one site reacted prevents ATPase activity in both F_1 and CF_1 suggests they are close together (Deters et al., 1975; Ferguson et al., 1975a,b; Cantley and Hammes, 1975a). The location and distance between the quercetin sites is uncertain although they must be on the α or β subunits (Deters et al., 1975). The NBPM sites are located on the γ subunit. The distance between these sites is uncertain although they would be expected to be close together if the cysteine groups form a disulfide bond. Experiments to measure the distance between these sites are currently underway. The subunit locations and distance between the tight nucleotide sites are still not known. The results obtained thus far permit a quite detailed picture of the topographical structure of CF_1 to be developed; experiments directed at amplification and refinement of this structure are now in progress.

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