Resonance Energy Transfer between Tryptophan 57 in the ϵ Subunit and Pyrene Maleimide Labeled γ Subunit of the Chloroplast ATP Synthase[†]

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ABSTRACT: The intrinsic fluorescence of the catalytic portion of the chloroplast ATP synthase (CF1) is quenched when cysteine 322, the penultimate amino acid of the γ subunit, is specifically labeled with pyrene maleimide (PM). The ϵ subunit of CF1 contains the only two residues of tryptophan, which dominate the intrinsic fluorescence of unlabeled CF1. CF1 deficient in the ϵ subunit (CF1-ϵ) was reconstituted with mutant ϵ subunits in which phenylalanine replaced tryptophan at position 15 (ϵW15F) and position 57 (ϵW15/57F). CF1(ϵW15F) containing a single tryptophan, ϵW57, was labeled with PM at γC322. Resonance energy transfer (RET) from ϵW57 to PM on γC322 occurred with an efficiency of energy transfer of 20%. RET was also observed from ϵW57 to PM attached to the disulfide thiols of the γ subunit (γC199,-205) with an efficiency of approximately 45%. The R_o (the distance at which the efficiency of energy transfer is 50%) for the ϵW57 and PM donor/acceptor pair is 30 Å, indicating that both γC322 and γC199,-205 must be within 40 Å of ϵW57. These RET measurements show that both γC322 and γC199,205 are located near the base of the α/β hexamer. This places the C-terminus of CF1 γ much closer to ϵ than hypothesized based on homology to crystal structures of mitochondrial F1. These new RET measurements also allow the alignment of the predicted ϵ subunit structure. The orientation is similar to that predicted from cross-linking and mutational studies for the ϵ subunit of *Escherichia coli* F1.

The synthesis of ATP in chloroplasts occurs at the expense of an electrochemical proton gradient generated by light-dependent electron transport. ATP synthase, CF1CFo,[1] couples the translocation of protons to the synthesis and hydrolysis of ATP. CF1 contains the nucleotide binding domains of the ATP synthase and is responsible for catalytic activity, whereas CFo is a hydrophobic membrane complex that permits the flow of protons across the thylakoid membrane. CF1 is hydrophilic and can be detached from thylakoid membranes and purified. In solution, CF1 is an active ATPase. CF1 is composed of nine individual polypeptide chains of five distinct types in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ [for recent reviews, see (*1*, *2*)].

The catalytic activity of CF1CFo is strictly regulated to limit ATP hydrolysis under conditions that do not favor ATP synthesis. Illumination of thylakoid membranes causes the formation of an electrochemical proton gradient that in turn stimulates the release of the inhibitory bound ADP and relieves the inhibition by the ϵ subunit. Reduction of the γ disulfide (γC199,205) further stimulates ATP synthesis rates (*3*). In the absence of the proton gradient, ADP binding, oxidation of the γ disulfide, and reimposition of the inhibition by the ϵ subunit all act to prevent ATP hydrolysis (*4*).

The ϵ subunit is a 14.7 kDa protein comprised of 134 amino acids. The location of ϵ within CF1 has been determined by resonance energy transfer (RET). Seven different sites within CF1 have been specifically labeled with fluorescent probes, and resonance energy has been observed between these sites and a cysteine within ϵ labeled with a fluorophore (ϵC6) (*5*). All of these measurements have converged to a location of ϵC6 that places ϵ under the $\alpha\beta$ hexamer ring and closely associated with the γ subunit (*6*). Although the predominant association between the ϵ subunit and CF1 appears to be through the γ subunit, the mechanism by which the ϵ subunit regulates activity is not understood. It is, however, clear that perturbations in the γ subunit, such as reduction of the γ disulfide bond, affect the binding of the ϵ subunit (*7*).

The ϵ subunit is an inhibitor of ATP hydrolysis of both CF1CFo and CF1 in solution. Truncations of either the amino terminus or the carboxyl terminus diminish the ability of the ϵ subunit to inhibit ATP hydrolysis by CF1 in solution (*8*). The ϵ subunit is also required to block the nonproductive flow of protons through CF1CFo. Thylakoid membranes containing CF1 depleted of ϵ (CF1-ϵ) are permeable to protons, and this permeability is prevented upon addition of free ϵ to the membranes (*9*).

The structural study of ϵ interactions is aided by X-ray crystal structures of portions of the mitochondrial F1 (MF1) and of the ϵ subunit from *Escherichia coli*. Although the MF1 structures (*10*, *11*) did not resolve much of the γ subunit or either the δ or ϵ subunits, they did show the $\alpha\beta$ hexamer ring. The amino acid sequences of the α and β subunits of MF1, *E. coli* F1, and CF1 are highly conserved (*2*). The

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[1] Abbreviations: BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; CF1, chloroplast coupling factor 1; CFo, hydrophobic portion of the chloroplast ATP synthase; F1, coupling factor of ATP synthase; NEM, *N*-ethylmaleimide; PM, *N*-(1-pyrene)-maleimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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structure of the *E. coli* ϵ subunit has been determined by both NMR (*12*) and X-ray diffraction (*13*). *E. coli* ϵ is composed of 138 amino acids and also inhibits ATP hydrolysis. The aligned primary sequences of CF1 ϵ and *E. coli* ϵ have a 25% identical occurrence of residues, with an additional 46% of the residues showing similarity. The position of the ϵ subunit within *E. coli* F1 has been determined by both mutational analysis (*14*) and direct cross-linking (*15*). Given the similarity of these subunits, the structure and orientation of CF1 ϵ is predicted to be similar to its *E. coli* counterpart.

Comparison of the γ subunit structure of MF1 to the locations of sites on the γ subunit of CF1 determined by RET shows an important difference. The carboxyl terminus of the γ subunit of MF1 was shown to extend up the center of the $\alpha\beta$ hexamer with the carboxyl terminus of γ located near the top of the hexamer. This is in stark contrast to the resonance energy transfer data, which position the penultimate cysteine of the CF1 γ subunit (γC322) at the base of the $\alpha\beta$ ring, more than 60 Å away from the location resolved in the MF1 structure. The carboxyl terminus of the γ subunit of MF1 is involved in hydrophobic interactions with the upper portion of the $\alpha\beta$ ring and was proposed (*10*) to act as a spindle for the rotation of γ within the $\alpha\beta$ ring during catalysis. If the carboxyl terminus of the γ subunit in CF1 is located below the $\alpha\beta$ ring, the catalytic mechanism of CF1 would not require the spindle suggested in MF1. This possibility has recently been investigated by C-terminal truncations of the CF1 γ (*16*).

This paper describes RET between a tryptophan at position 57 of the ϵ subunit of CF1 and pyrene maleimide attached to two specific sites within the γ subunit. The data were used to position the ϵ subunit within CF1. The ϵ subunit possesses the only two tryptophan residues within CF1. Mutation of tryptophan at position 15 to phenylalanine (ϵW15F) has allowed the use of the remaining tryptophan (ϵW57) as a specific fluorescence probe within CF1.

MATERIALS AND METHODS

Generation of ϵ Mutations. All ϵ mutant plasmids and inclusion bodies were prepared according to McCarty and Cruz (*17*). The ϵC6S mutation was shown to retain native activity and folding (*18*). The mutation ϵW15F was constructed from the ϵC6S plasmid using unique site elimination mutagenesis (Clontech, Palo Alto). The forward primer for W15F was, from 5′ to 3′, CTTTTACTTCTGAATTGAAAATACTTCGATTC. The reverse primer altered a unique *Xho*I cut site to *Pvu*II using the primer GACTGCTTTACCGCAGCTGCCTCGCGCG. The mutation ϵW15/57F was constructed by converting the *Pvu*II cut site back to *Xho*I with the reverse primer GCTTTACCGCTCGAGCCTCCCGCG and incorporating the forward primer for the W57F mutation GAGCCAATGTTAAGAATTGGTCGTTAAG.

Reconstitution of CF1 with Modified ϵ. CF1 (*7*) and CF1-ϵ (*19*) were prepared according to established methods. Prior to reconstitution of CF1-ϵ with recombinant ϵ, approximately 20 mg of CF1-ϵ stored as an ammonium sulfate precipitate was centrifuged at 16000*g* for 5 min at 25 °C. The pellet was dissolved in 20 mM Tris-HCl (pH 8.0) with 100 μM $CuCl_2$ to ensure complete oxidation of the γ disulfide. CF1-ϵ was then passed through two consecutive 3 mL Sephadex G-50 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0) (*20*). The protein concentration of CF1-ϵ was estimated by the Bradford method (*21*). Inclusion bodies containing the ϵ subunit were incubated for 15 min with 30 units of DNase I (Boehringer Mannheim) in 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$ at 37 °C, and the DNase was removed by centrifugation at 16000*g* for 5 min. Inclusion bodies (approximately 2−4 mg) containing the ϵ subunit were solubilized in 8 M urea, 20 mM Tris-HCl (pH 8.0). The pellets were incubated in the Tris−urea solution for 2 h at room temperature and then centrifuged at 16000*g* for 5 min to remove insoluble material. The protein concentration of unfolded ϵ was estimated by the Bradford method as corrected by Richter et al. (*5*) and adjusted to 2 mg/mL in Tris−urea.

The unfolded ϵ subunit in the Tris−urea solution was diluted to 0.2 mg/mL (10-fold dilution) in 20% ethanol, 30% glycerol solution with 20 mM Tris-HCl (pH 8.0) and then reconstituted with CF1-ϵ such that the final solution contained a 6:1 ratio (mole:mole) of ϵ to CF1-ϵ and the ethanol concentration was below 7%. Reconstituted CF1 was removed from excess ϵ by passage through a Sephadex G-75 column (4 times the volume of reconstitution volume) equilibrated with 20 mM Tris-HCl (pH 8.0). CF1 elutes in the void volume of this column. The void volume fractions were then passed through a 2 mL DEAE-cellulose (Whatman) column equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with approximately 5 column volumes of 20 mM Tris-HCl (pH 8.0), 40 mM NaCl followed by elution of CF1 with 20 mM Tris-HCl (pH 8.0), 400 mM NaCl. Reconstituted CF1 was stored as an ammonium sulfate precipitate at 4 °C in the presence of 1 mM ATP until ready for use. The content of ϵ in CF1 was estimated by tryptophan fluorescence after complete degradation of the CF1 complex with papain and the fluorescence compared to a tryptophan standard (*22*). Ca^{2+}-ATPase activity of CF1 was measured in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, and 5 mM $CaCl_2$ at 37 °C for 2−5 min. The amount of phosphate produced was measured colorimetrically (*23*).

Fluorescence Lifetime Measurements. CF1 stored as an ammonium sulfate precipitate was pelleted and dissolved in 20 mM Tris-HCl (pH 8.0). Two consecutive passes through 3 mL Sephadex G-50 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0) were used to remove residual ammonium sulfate. CF1 was diluted in 20 mM Tris-HCl (pH 8.0) to approximately 3 μM to minimize absorbance by the sample. Fluorescence lifetime measurements were made using a single photon counting apparatus with a picosecond synchronously pumped mode-locked dye laser (*24*). Instrument resolution was 65 ps. Time-resolved fluorescence involves the monitoring of the fluorescent decay of a sample as a function of emission wavelength and using the decay of fluorescent intensity, $I(t)$, to calculate the component lifetimes of the sample:

$$I(t) = \Sigma_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i is the amplitude of the fluorescence signal of component lifetime τ_i. Data analysis was performed using TCphoton software (D. Toptygin, proprietary software). All measurements were made at 25 °C.

Labeling of CF1 with Pyrene Maleimide. Desalted CF1 in 20 mM Tris-HCl (pH 8.0) was adjusted to give 1−3 mg

of protein in 600 μL. For labeling CF1 at γC322, the solution was then split into two 300 μL fractions. *N*-(1-Pyrene)-maleimide (Molecular Probes) in DMF at an approximately 1:1 (mole:mole) ratio of CF1 to PM was then added to one fraction and incubated for 30 s. As a control, an equivalent amount of DMF was added to the second fraction. The samples were immediately passed through two consecutive 3 mL Sephadex G-75 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0).

CF1 labeled with PM at the γ subunit disulfide was prepared as follows. To desalted, reconstituted CF1 (1−3 mg of protein in 600 μL) was added *N*-ethylmaleimide to a final concentration of 2 mM, and the solution was allowed to sit for 30 min at room temperature to block accessible thiols in the enzyme. Solid DTT was added to a final concentration of 50 mM, and the solution was incubated for 2 h. The reduced CF1 was passed through two 3 mL Sephadex G-50 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0). The solution was diluted to 600 μL, and the resulting concentration was estimated by the Bradford method (*21*) as quickly as possible. The CF1 solution was split into two 300 μL fractions. To one fraction was added 3 μL of PM in DMF such that the final concentration of PM was 3 times that of the CF1. As a control, an equivalent amount of DMF was added to the second fraction. Both solutions were allowed to sit for 60 s, and then immediately passed through two consecutive 3 mL Sephadex G-75 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0).

All CF1 used in fluorescence measurements was protected from the light and diluted to 1 mL and immediately placed in a fluorometer for measurements. After the fluorescence measurement was recorded, the protein content was determined, in quadruplicate, by the Lowry method (*25*). The concentration of PM was determined by the absorbance at 343 nm using an extinction coefficient of 37 500 M^{-1} cm^{-1} (*26*).

Resonance Energy Transfer Measurements. Fluorescence measurements were performed using either an Olis modified SLM Aminco-SPF-500C or a Shimadzu RF-5000 spectrofluorometer. All fluorescence measurements were performed at an excitation of 295 nm with the fluorescence emission monitored at 343 nm. Corrections were made in the fluorescence measurements for background, as measured by similarly treated CF1(ϵW15/57F), and protein content. The transfer efficiency (*E*) between tryptophan and pyrene was calculated based upon the fluorescence at 343 nm in the presence and absence of the pyrene acceptor molecule according to eq 2 where F_D is the fluorescence of the donor (unlabeled CF1) and F_{DA} is the fluorescence of the donor in the presence of the acceptor (labeled CF1).

$$E = 1 - \frac{F_{DA}}{F_D} \qquad (2)$$

Extinction coefficients at 295 nm for reconstituted CF1 were calculated by linear regression of 295 nm absorbance measurements taken at a range of concentrations between 0 and 15 μM CF1. The extinction coefficient of ϵW57 was assumed to be the difference between the extinction coefficients of CF1(ϵW15F) and CF1(ϵW15/57F). All measurements were taken in triplicate and protein concentrations determined by the Lowry method (*25*).

The quantum yield of tryptophan was calculated by comparison of the extinction coefficient and the fluorescence spectrum of tryptophan taken on an SLM-48000, corrected for both instrument response and background fluorescence [as estimated by the fluorescence of CF1(ϵW15/57F)] to the fluorescence spectrum of quinine sulfate (*27*).

Partial Trypsinization and Immunoblotting of CF1. CF1(ϵW15F) labeled with PM at γC322 was incubated with 50 mM DTT in 20 mM Tris-HCl (pH 8.0) for 2 h to reduce the γ disulfide. DTT was removed by two consecutive passes through 3 mL Sephadex G-50 centrifuge columns equilibrated with 20 mM Tris-HCl (pH 8.0). Trypsin was added at a ratio of 1 mg per 100 mg of CF1 and reacted with CF1 at room temperature for a predetermined time. The reaction was stopped with the addition of 1:10 (v/v) 24% trichloroacetic acid, and the tubes were placed on ice. After 5 min, the samples were centrifuged at 16000*g* for 5 min. The supernatants were discarded, and the pellet was dissolved in 160 mM sodium bicarbonate with 1% SDS.

Protein electrophoresis was performed using a 15% acrylamide SDS−PAGE Tricine gel (*28*). Transfer to nitrocellulose was performed using a Bio-Rad Mini Trans-Blot cell. After transfer, proteins were fixed to the nitrocellulose using a 1% glutaraldehyde solution in 50 mM $NaHPO_4$ (pH 7.5). Nitrocellulose was probed using rabbit sera from Covance (Denver, PA) prepared against reduced bovine serum albumin reacted with the cross-linker *N*,*N*′-1,5-naphthalenebismaleimide (NBM). Secondary antibody was goat anti-rabbit IgG−peroxidase conjugate (Sigma) and was developed using an ECL detection kit (Amersham Pharmacia).

RESULTS

Fluorescence of CF1 Is Quenched by PM. Spinach CF1 contains 11 residues of the amino acid cysteine. Each α and β subunit as well as the ϵ subunit possesses a single cysteine, and the γ subunit contains the remaining four cysteines. Two of the γ subunit cysteines are involved in a disulfide linkage (γC199,γC205); this disulfide is the only disulfide in CF1. Thus, all other cysteines are theoretically available for reaction with cysteine-specific reagents under oxidizing conditions. In practice, however, the reactivity of these cysteines varies widely. The α and β cysteines in native CF1 have very low reactivity. The ϵ subunit can be labeled at ϵC6 either in isolated ϵ or by prolonged exposure of CF1 to a reagent (*5*). Of the two remaining γ cysteines, γC89 is inaccessible to sulfhydryl reagents unless thylakoid membranes are illumunated in the presence of the reagent (*29*). γC322 is the most reactive cysteine in spinach CF1, and both soluble and membrane-bound CF1 are readily labeled (*30*). This reactive cysteine is not conserved among chloroplast ATP synthases, and its alkylation has no effect on ATP synthesis or hydrolysis.

Only two residues of tryptophan are present in spinach CF1, and both are located within the ϵ subunit. Although tyrosine and phenylalanine also fluoresce, using an exciting wavelength of 295 nm and monitoring emission above 310 nm it is possible to monitor the fluorescence of tryptophan without interference from that of tyrosine and phenylalanine.

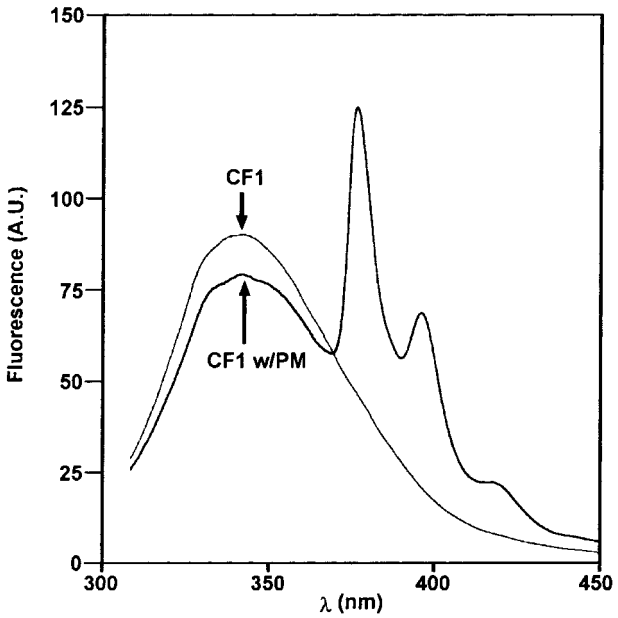

FIGURE 1: Fluorescence emission spectra of CF1 (approximately 1.5 μM) when excited at 295 nm. Spectra show CF1 and CF1 covalently bound with PM at γC322 (approximately 50%). Fluorescence spectra are corrected for protein content and taken under identical conditions at 25 °C.

γC322 can be specifically labeled with a cysteine-specific probe and any alteration in the tryptophan fluorescence of CF1 in solution caused by this probe detected. For this purpose, pyrene maleimide (PM) was used to label CF1. PM has both a very high specificity for cysteine residues and its absorbance maximum of 343 nm is in the range of tryptophan emission. Because of these traits, if PM is in proximity of tryptophan, resonance energy transfer (RET) is possible from tryptophan to PM, causing a quenching of tryptophan fluorescence. Labeling of CF1 with PM was carried out at very short reaction times and dilute concentrations of PM due to its low solubility in water and high hydrophobicity, which increase its reactivity with buried cysteines (such as ϵC6).

About half of the CF1 is labeled with PM by this procedure. Using higher concentrations of PM or extending the incubation time increases PM incorporation, but PM also labels ϵC6 (data not shown). For this reason, no data were taken with more than approximately half of the CF1 labeled with PM. The tryptophan fluorescence of CF1, however, is decreased by the PM labeling (Figure 1). Although this result is consistent with energy transfer from tryptophan to PM, the specificity of PM for γC322 and specific quenching of tryptophan fluorescence must be demonstrated.

PM Labeling of CF1 Is Specific to γC322. The specificity of PM for γC322 was shown by immunoblotting of CF1 using a rabbit antibody raised against reduced BSA labeled with naphthalenebismaleimide (NBM). This antibody reacts with CF1 labeled with NBM (Evron, unpublished data), and with PM (Figure 2, lane e), but does not detect unlabeled CF1 (Figure 2, lane d). Moreover, the anti-NBM antibody detects less than 1 pmol of PM bound to CF1 (data not shown). The γ subunit is the only subunit in PM-labeled CF1 in which the anti-NBM antibody detects bound PM.

To ensure that the γ cysteine labeled with PM is γC322, limited proteolysis was used. Trypsin cleaves the γ subunit

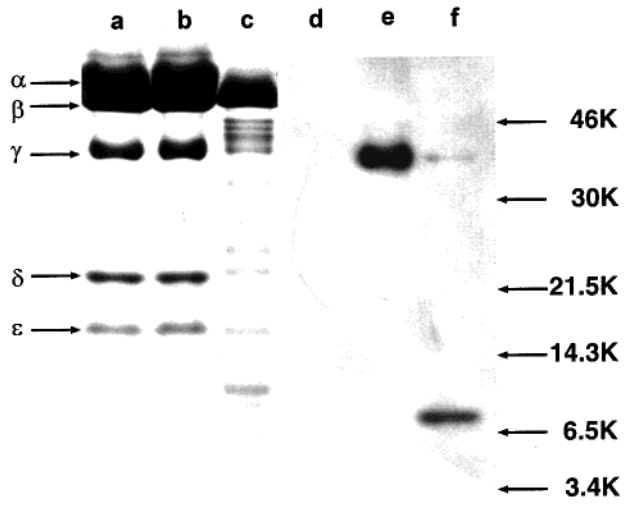

FIGURE 2: Limited trypsinization of CF1 (approximately 8 μg/lane). Lanes a−c are Coomassie-stained CF1 before labeling with PM (a), after labeling (b), and after labeling followed by trypsinization for 20 min (c). Lanes d−f are the same samples as lanes a−c but probed with anti-NBM antibody.

of CF1 into three large polypeptides that remain associated with the α/β hexamer (*31*). The tryptic fragment containing γC322 had a mass of 10 kDa and contains a single cysteine (γC322). After limited trypsinization, PM-labeled CF1 shows a single additional band at approximately 10 kDa that is readily detected by immunoblotting with the anti-NBM antibody (Figure 2, lane f). Thus, the only cysteine labeled with PM by the procedure used to generate the labeled CF1 in Figure 1 is γC322.

Reconstitution of Modified CF1. Mutations of the ϵ subunit were constructed that specifically altered tryptophans to phenylalanines. These mutants were based on the original ϵ mutation incorporating a substitution of the sixth residue from cysteine to serine. This root mutation (ϵC6S) shows wild-type activity (*8*) and is beneficial in these experiments both in preventing dimerization of the ϵ subunit prior to reconstitution as well as in obviating the risk of pyrene maleimide (PM) binding to the ϵ subunit. Mutant ϵ subunits in which the tryptophans, ϵW15 and ϵW57, of the ϵ subunit were replaced with phenylalanines either individually or together were generated. The mutation, ϵW15F, was selected for RET measurements because the remaining tryptophan, ϵW57, was shown to contribute the predominant fluorescence lifetime within CF1 (Table 1).

Reconstitution of CF1-ϵ with mutant ϵ results in CF1 with ATPase activity identical to that of native CF1. CF1, CF1-(ϵW15F), and CF1(ϵW15/57F) ($n = 3$ for each type, $n = 9$ total) showed calcium-dependent ATP hydrolysis rates of 1.2 ± 0.4 μmol of P_i min^{-1} (mg of protein)$^{-1}$, and CF1-ϵ showed a rate of 11.9 ± 0.6 μmol of P_i min^{-1} (mg of protein)$^{-1}$ ($n = 6$). Thus, each of the mutant ϵ subunits is active as an ATP inhibitor. The extent of reconstitution for ϵW15F with CF1-ϵ was also examined by determining the tryptophan content (*22*) of both CF1(ϵW15F) and CF1(ϵW15/57F), which showed CF1(ϵW15F) contained 1.0 ± 0.2 ($n = 5$) mol of tryptophan/mol of CF1 relative to CF1(ϵW15/57F).

Resonance Energy Transfer Using ϵW57. The intrinsic fluorescence of CF1(ϵW15F) excited at 295 nm was quenched after incubation of the enzyme with PM under the same conditions used for native CF1 (Figure 3). The fluorescence

Table 1: Lifetime Analysis at 343 nm[a] of CF1 with and without Pyrene Maleimide

protein	τ_1[b]	τ_2[b]	τ_3[b]	τ_4[b]	α_1	α_2	α_3	α_4	$<\tau>$[b,c]	χ^2
CF1	0.08	0.7	2.7	7.7	0.20	0.19	0.32	0.30	3.3	1.119
CF1 w/PM[d]	0.07	0.6	2.6	7.6	0.24	0.19	0.30	0.27	3.0	1.011
CF1(ϵW15F)	0.07	0.7	2.7	7.5	0.24	0.16	0.15	0.45	4.0	1.049
CF1(ϵW15F) w/PM[d]	0.04	0.5	2.4	7.5	0.31	0.15	0.14	0.40	3.4	1.006
CF1(ϵW15/57F)	0.05	0.5	2.2	6.2	0.50	0.20	0.19	0.11	1.2	1.417
CF1(ϵW15/57F) w/PM[d]	0.04	0.5	2.1	6.2	0.54	0.20	0.17	0.09	1.0	1.525

[a] Monitored emission with 295 nm excitation. [b] Lifetime in nanoseconds. [c] Average lifetime is calculated as $<t>=\sum\alpha_i\tau_i/\Sigma\alpha_i$. [d] Samples approximately 50% labeled.

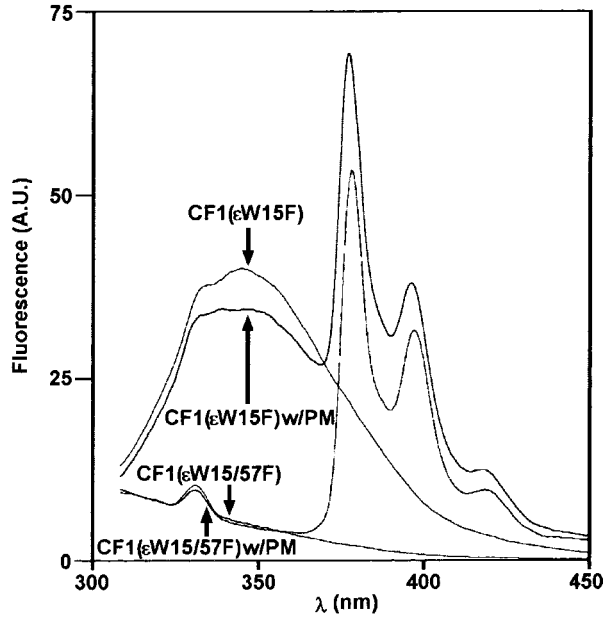

FIGURE 3: Fluorescence emission spectra of CF1(ϵW15F) and CF1(ϵW15/57F) (approximately 0.75 μM) when excited at 295 nm. Spectra show CF1(ϵW15F) and CF1(ϵW15/57F) alone and covalently bound with PM at γC322. CF1(ϵW15F) is bound with approximately 65% PM, and CF1(ϵW15/57F) is bound with approximately 75% PM. Fluorescence spectra are corrected for protein content and taken under identical conditions at 25 °C.

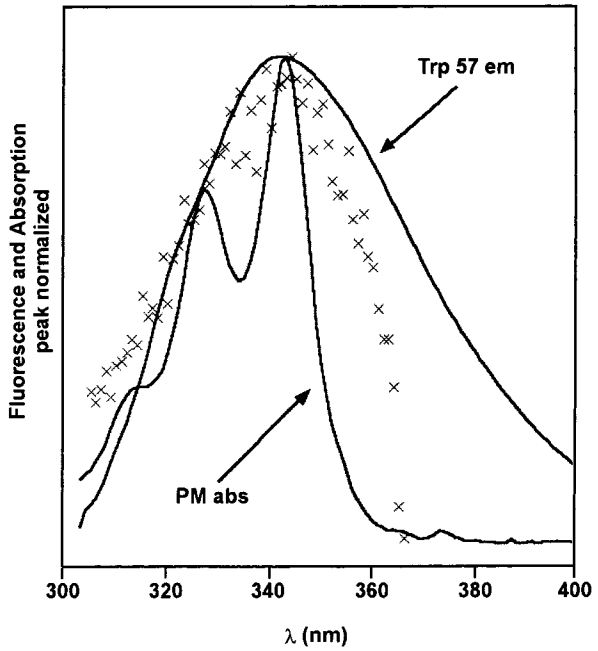

FIGURE 4: Spectral overlap between ϵW57 and PM. The fluorescence spectrum is the result of the subtraction of the fluorescence spectrum of CF1(ϵW15/57F) from the fluorescence spectrum of CF1(ϵW15F); both spectra have been corrected for instrument response and protein content. The absorbance spectrum is the result of the spectrum of CF1(ϵW15F) subtracted from the spectrum of CF1(ϵW15F) labeled with PM at γC322, both corrected for protein content. The symbol (×) denotes the difference between the fluorescence of CF1(ϵW15F) and CF1(ϵW15F) with PM at γC322 at whole wavelengths between 305 and 365 nm. Both spectra and the difference points have been peak-normalized.

of CF1(ϵW15/57), also excited at 295 nm, shows very low fluorescence with only slight change when labeled with PM (Figure 3).

Figure 4 shows the absorption spectrum of pyrene maleimide bound to CF1 superimposed on a fluorescence emission spectrum of ϵW57 generated by subtracting the fluorescence spectrum of CF1(ϵW15/57F) from the fluorescence spectrum of CF1(ϵW15F). Shown in Figure 4 are the values at whole wavelengths between 305 and 365 nm of the subtraction of a spectrum of PM-labeled CF1(ϵW15F) from one of unlabeled CF1(ϵW15F). The difference spectrum shows that the quenching of tryptophan follows the fluorescence spectrum of ϵW57. This is consistent with the quenching of ϵW57 occurring through resonance energy transfer with PM.

The fluorescence decay kinetics of CF1, both unlabeled and labeled with PM (0.55 mol/mol), were examined with excitation at 295 nm and emission at the peak of tryptophan emission (343 nm). The preexponential factor (α_i) associated with each lifetime shows the relative population of molecules exhibiting that lifetime. The amplitude average lifetime, $<\tau>$, weights each lifetime according to that lifetime's contribution to the total population of molecules. In Table 1, $<\tau>$ at 343 nm decreased when PM was present on CF1. This fact is consistent with energy transfer from ϵW57 to PM.

A second attribute of resonance energy transfer is the increase in acceptor (PM) fluorescence. Investigation of the acceptor fluorescence is hampered by the presence of the donor fluorescence at the same wavelength. Investigation of the lifetimes of the acceptor was also hampered by the fact that pyrene does have a small absorbance at the excitation wavelength (295 nm). This means that the lifetimes associated with PM are the result of both direct excitation and energy transfer. This coupled with the overlapping fluorescence of tryptophan and PM makes acceptor fluorescence uninterpretable for energy transfer (*32*).

The specificity of PM labeling of CF1(ϵW15F) was determined by immunoblotting using the anti-NBM antibody (Figure 5). As was the case for native CF1, PM labeled CF1(ϵW15F) specifically at γC322. ATP hydrolysis was unaffected by PM labeling on all types of CF1. Labeled native CF1, CF1(ϵW15F), and CF1(ϵW15/57F) all gave the same

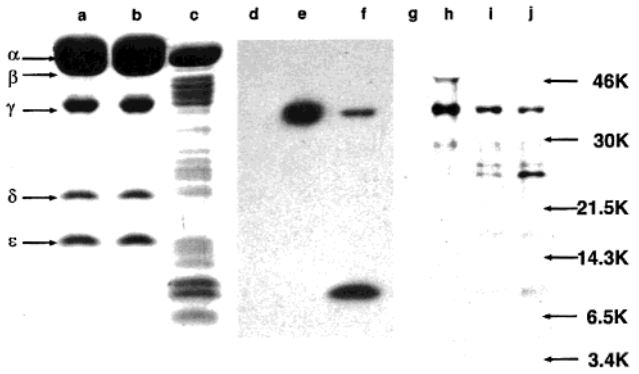

FIGURE 5: Limited trypsinization of CF1 (approximately 8 μg/lane). Lanes a−c are Coomassie-stained CF1(ϵW15F) before labeling with PM (a), after labeling (b), and after labeling followed by trypsinization for 20 min (c). Lanes d−f are the same samples as lanes a−c but probed with anti-NBM antibody. Lanes g−j are treated the same as lanes d−f but contain CF1(ϵW15F) blocked with NEM (g), blocked and labeled with PM at γC199,205 (h), blocked, labeled, and trypsinized for 30 s (i), and blocked, labeled, and trypsinized for 60 s (j). Coomassie-stained samples of lanes g and h were identical to lanes a and b.

calcium-dependent ATP hydrolysis rate of 1.2 ± 0.3 μmol of P_i min^{-1} (mg of protein)$^{-1}$ ($n = 2$ for each type, $n = 6$ total), the same rate as was seen for unlabeled CF1.

The amount of fluorescence quenching seen by PM is related to the amount of tryptophan in CF1, and the CF1 devoid of tryptophan CF1(ϵW15/57F) shows almost no fluorescence signal when excited at 295 nm, indicating that emissions above 310 nm are due solely to the tryptophan found in CF1. Since this quenching of fluorescence corresponds to the fluorescence spectrum of ϵW57 and the quenching affects the lifetime of the fluorescence, this fluorescence quenching is due to resonance energy transfer from tryptophan as the donor to pyrene maleimide labeled only at γC322 as the acceptor.

RET Shows γC322 Is Near the ϵ Subunit. The availability of CF1(ϵW15F) allows the calculation of a distance between PM and the sole tryptophan in CF1(ϵW15F), ϵW57. To use RET to determine a distance between ϵW57 and PM, the Förster distance, R_o, between these two molecules must be determined. The Förster distance is the distance between the two fluorophores at which 50% energy transfer would occur and is based upon physical characteristics of both fluorophores. Determination of R_o is based on the following equation (*27*):

$$R_o = (8.79 \times 10^{-5})(\kappa^2 J Q_D \eta^{-4})^{1/6} \quad (3)$$

Two important experimental values on which R_o is based are the spectral overlap (J) between the fluorescent emission of the donor and the absorbance of the acceptor and the quantum yield of the donor (Q_D). The spectral overlap was calculated by numerical integration of the spectra shown in Figure 4 and was calculated to be 1.57×10^{14} (nm^4 M^{-1} cm^{-1}). The quantum yield of ϵW57 was calculated by comparison of the extinction coefficient at 295 nm and the numerical integration of the emission spectrum of ϵW57 against the known standard, quinine sulfate. This resulted in a Q_D for ϵW57 of 0.33 and an R_o of 30 Å. This value is higher than a previously published value (20 Å) for R_o between PM and tryptophan (*33*). However, that value was based upon a tryptophan with a much lower Q_D, and 30 Å is within the range of possible Förster distances involving tryptophan (*27*). The index of refraction (η) was assumed to be 1.4, and κ^2 was assumed to be 2/3 (*34*).

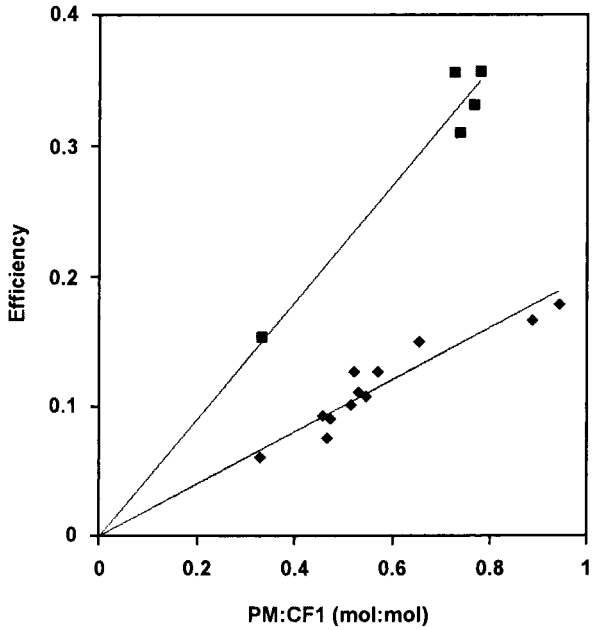

FIGURE 6: Fluorescence quenching of repetitive preparations of CF1(ϵW15F) covalently bound with PM at either γC199,205 (■) or γC322 (◆). Each data point represents an individual experiment as described under Materials and Methods.

The γ subunit disulfide has been determined to be in the proximity of the ϵ subunit by several methods (*5*, *35*). Given that the diameter of the CF1 is approximately 100 Å as determined by electron microscopy (*36*), the γ disulfide is probably no more than 40 Å away from the ϵ subunit. The disulfide (γC199,205) can be labeled with PM by first blocking γC322 with NEM and then reducing the disulfide. The specificity of PM for γC199,205 was seen by immunodetection of partially trypsinized CF1(ϵW15F). Unlike γC322, upon partial trypsinization, both γC199 and γC205 remain on a 27 kDa fragment; this fragment is quickly digested by trypsin to a 25 kDa fragment which contains only γC199, the other cysteine being lost into solution during trypsinization (*31*). Lanes g−i of Figure 5 show that the anti-NBM antibody detected the PM on 27 and 25 kDa fragments. Thus, at least one cysteine of the disulfide is labeled with PM. Because the two cysteines are close enough to form a disulfide bond, they are both considered to occupy the same position in terms of RET.

Several attempts were made to observe RET from ϵW57 to PM attached to γC199,205. In all cases, a strong quenching of fluorescence was observed, and the CF1 was typically about 80% labeled by PM. An additional labeling was performed at substoichiometric ratios of PM to CF1 to test the linearity of efficiency versus PM stoichiometry. The efficiency of energy transfer (Figure 6, squares) extrapolated to 100% efficiency is between 0.49 and 0.41 at 95% confidence, assuming the linearity of the regression. Energy transfer efficiency (E) is related to distance (R) by eq 4.

$$R = \sqrt{\frac{R_o^6(1 - E)}{E}} \quad (4)$$

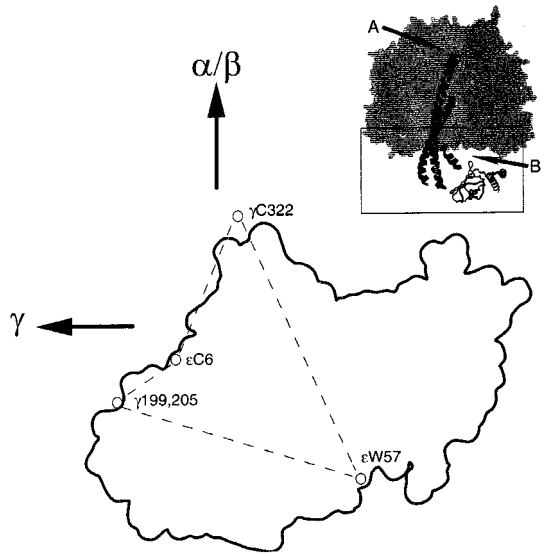

FIGURE 7: Approximate alignment of the *E. coli* ϵ structure using CF1 RET map data. The inset shows the relative position of ϵ to MF1 structure shown in cross section. MF1 oriented to CF1 RET map according to McCarty et al. (*2*). Inset labels are A, location of γ C-terminus in MF1 structure; and B, location of γ C-terminus according to CF1 RET data.

The extrapolated efficiency of energy transfer corresponds to a distance between ϵW57 and γC199,205 of 30−32 Å.

Similar measurements were performed using CF1(ϵW15F) labeled with PM at γC322 (Figure 6, diamonds). Because CF1(ϵW15F) incorporates the mutation ϵC6S, higher labeling was possible than in native CF1. Higher labeling was obtained by repeating the labeling procedure 2−3 times for a sample before measuring its fluorescence. When the labeling is extrapolated to 100%, the efficiency is calculated to be 0.2 with a 95% confidence between 0.17 and 0.22. The calculated distance between ϵW57 and γC322 is between 40 and 36 Å.

RET Orientation of CF1 ϵ Agrees with E. coli ϵ Orientation. The orientation of the ϵ subunit within CF1 is unknown since only a single location had previously been positioned within CF1 by RET measurements (ϵC6). The calculation of RET distances between ϵW57 and both γC322 and γC199,205 gives a second location within ϵ which has now been related to the existing RET map of CF1. By using a distance between ϵC6 and ϵW57 estimated from the distance between the analogous residues located in the X-ray diffraction structure of the *E. coli* ϵ subunit (*13*), it has been possible to constrain the possible locations of the ϵ subunit within CF1.

Figure 7 shows the *E. coli* ϵ subunit structure oriented with respect to the α, β, and γ subunits. This orientation is based upon the location of ϵC6 (ϵD7 in the *E. coli* structure) within the RET map and positioned to orient ϵW57 (ϵE59 in the *E. coli* structure) relative to γC322 and γC199,205 as suggested by the RET data presented in this paper. The amino acid sequence similarity of the ϵ subunit of CF1 to that of *E. coli* F1 suggests that these proteins have similar structures. Studies involving cross-linking and mutational analysis between the ϵ and γ subunits of *E. coli* have positioned the *E. coli* ϵ in roughly the same orientation as the ϵ oriented in Figure 7 (*37*). Both orientations position one face of the N-terminal β-barrel toward the γ subunit in the middle of CF1 with the C-terminal α-helices facing away from the central core. Both structures orient the conserved histidine (ϵH37 in CF1 numbering) at the bottom of CF1. The top inset in Figure 7 shows the orientation of *E. coli* ϵ relative to the partial crystal structure of MF1 according to the estimated orientation of both the ϵ and MF1 structures to the CF1 RET map (*2*). Arrow A shows the position of the γ C-terminus as determined by the MF1 structure; arrow B shows the γ C-terminus as determined by the CF1 RET map and resonance energy transfer to ϵW57. The difference between these two points is over 60 Å. If the γ C-terminus of CF1 were in the position of the γ C-terminus seen in the MF1 structure, the PM labeled to γC322 would have been far beyond the distance possible for resonance energy transfer. Thus, the data generated from this work support the assertions both that the *E. coli* ϵ subunit and CF1 ϵ subunit share common structure and positioning within ATP synthase and that the existing CF1 RET map correctly positions the γ C-terminus as located by γC322.

DISCUSSION

Previous use of RET has generated a group of specific locations within spinach CF1 encompassing 12 sites within the α, β, γ, ϵ [reviewed in (*38*)], and most recently δ (*39*) subunits. Using the distances determined between various combinations of these locations, a RET map has been generated which locates these unique sites relative to each other (*6*, *39*). Resonance energy transfer to two locations within the γ subunit has shown γ to be located in the center of CF1. The γ subunit is predicted to coordinate the catalysis of F1 by altering its orientation relative to the catalytic β nucleotide binding sites during enzymatic turnover, and this movement has been proposed to be conferred by the complete rotation of the γ subunit (*40*). The center of rotation for the γ subunit is assumed to be a long spindle composed of the N- and C-terminal ends of the γ subunit. The RET coordinates for γC322 suggest that the C-terminus of the γ subunit of CF1 is not involved in such a spindle. The location of CF1 γC322 relative to the ϵ subunit therefore provides important clues as to the applicability of these ideas to CF1.

Using native CF1, we have shown that PM specifically labels γC322 and that this labeling quenches CF1 fluorescence. Using CF1 devoid of tryptophan, we have shown that no quenching is observed if tryptophan is absent. Using CF1 containing a single tryptophan, we have shown that the quenching is caused by energy transfer from tryptophan within the ϵ subunit to PM attached to γC322. These results are in agreement with previous RET data and are verified by the observation of RET between PM labeled to γC199,-205 and ϵW57. Estimated distances between ϵW57 and both γC322 and γC199,205 position the *E. coli* structure relative to the CF1 RET map in a similar orientation as seen in *E. coli* F1 for ϵ relative to the α, β, and γ subunits.

The presence of energy transfer between ϵW57 and γC322 shows that in CF1 the C-terminus of the γ subunit is indeed located near the base of the αβ ring, as was shown in previous resonance energy experiments (*5*, *30*, *41*). The distances calculated based on this energy transfer show ϵ in a position and orientation that are in rough agreement with previous data from both *E. coli* F1 and CF1 studies. This position of the CF1 γ C-terminus is inconsistent with that

of the MF1 γ C-terminus. The X-ray structure of beef heart MF1 shows that the C-terminus of the γ subunit is located near the top of a long α-helix which extends through the center of the $\alpha\beta$ hexamer. In CF1, as many as 14 amino acids may be deleted from the C-terminus of the γ subunit of CF1 without loss of the ability of the γ subunit to associate with $\alpha_3\beta_3$ from CF1 or of the ATPase activity in the reconstituted $\alpha_3\beta_3\gamma$ complexes (*16*). These results are clearly inconsistent with the notion that the C-terminus of CF1 γ extends up through the $\alpha\beta$ hexamer.

Sokolov et al. (*16*) proposed that the C-terminal region of the γ subunit of CF1 could fold back toward the center of the enzyme. The RET measurements reported in this paper as well as those published previously (*6*) are consistent with this suggested location of the C-terminus of CF1 γ. Although it remains to be proven, it is very likely that γC322 can be cross-linked to γC89 (*42*). The MF1 γ residue analogous to CF1 γC89 is located near the base of the $\alpha\beta$ hexamer. RET measurements of CF1 place γC89 in a similar position. Thus, the cross-linking of γC89 and γC322 would place the C-terminus of CF1 γ close to the bottom of the $\alpha\beta$ hexamer.

In conclusion, the results of this study have shown that the C-terminus of the γ subunit of CF1 is located within 40 Å of the CF1 ϵ subunit. Currently, 10 locations from the α (*41*), β, γ (*30*), δ (*39*), and ϵ (*5*) subunits of CF1 have shown detectable resonance energy transfer to γC322. Distances based upon these RET measurements all position γC322 in the location described in this paper. Conformations of the γ subunit therefore appear to differ between MF1 and CF1.

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