Asymmetry of the α Subunit of the Chloroplast ATP Synthase as Probed by the Binding of Lucifer Yellow Vinyl Sulfone†

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ABSTRACT: The catalytic portion of the chloroplast ATP synthase (CF1) is structurally asymmetric. Asymmetry of the otherwise symmetrical $\alpha_3\beta_3$ heterohexamer is induced by the presence of tightly bound nucleotides and interactions with the single-copy, smaller subunits. Lucifer Yellow vinyl sulfone (4-amino-*N*-[3-(vinylsulfonyl)phenyl]naphthalimide-3,6-disulfonic acid) rapidly and covalently binds to lysine 378 on one α subunit [Nalin, C. M., Snyder, B., and McCarty, R. E., (1985) *Biochemistry 24*, 2318−2324] [Shapiro, A. B. (1991) Ph.D. Thesis, Cornell University, Ithaca, NY). The asymmetrical binding of Lucifer Yellow to CF1 provides a method to investigate the cause of asymmetry in the α subunits. The reaction of CF1 with Lucifer Yellow was monitored by total fluorescence of bound Lucifer Yellow as well as by quantitative determination of Lucifer Yellow bound to the tryptic peptide that contains lysine 378 of the α subunit. The total binding of Lucifer Yellow to CF1 was not affected by the presence of tightly bound nucleotides or nucleotide in the medium. Neither the total binding of Lucifer Yellow to CF1 nor the reaction of α-lysine 378 with Lucifer Yellow was changed by the removal of the ϵ subunit, the δ subunit, or both subunits. The extent of incorporation of Lucifer Yellow into lysine 378 of the α subunit in $(\alpha\beta)n$ was about three times that of Lucifer Yellow incorporation into CF1. Reconstitution of $(\alpha\beta)n$ with γ restored the binding of one Lucifer Yellow per $\alpha_3\beta_3\gamma$. Therefore, the interactions between γ and the $\alpha\beta$ heterohexamer are important in conferring asymmetry to the α subunits of CF1.

Light absorption by chloroplast thylakoid membranes begins a process that drives the net movement of protons across the membrane. The movement of these protons through the chloroplast ATP synthase results in the formation of ATP from ADP and Pi. This large multisubunit enzyme has a similar mechanism and structure as ATP synthases from bacteria and mitochondria. Chloroplast ATP synthase consists of two discrete subunit groupings, called CF1[1] and CFo. CF1 contains the catalytic and regulatory portions of the enzyme, whereas CFo is the membrane-embedded proton channel and the point of attachment of CF1 to the thylakoid membrane. CF1 can be readily detached from the thylakoid membrane and studied in solution as an ATPase. CF1 comprises five different subunits, referred to as α, β, γ, δ, and ϵ in order of decreasing molecular weight, and has the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ (*1*).

The functions of the smaller CF1 subunits have been investigated by subunit depletion (*2*−*5*) and mutagenesis (*5*, *6*). The ϵ subunit has dual roles. It is required for proton gating by CF1 (*2*, *3*) and is involved in the regulation of activity (*7*). Removal of the ϵ subunit heightens the sensitivity of the γ subunit to trypsinization and reducing agents (*8*). The δ subunit is required for the functional binding of CF1 to CFo (*4*). The δ subunit also stabilizes the $\alpha_3\beta_3\gamma$ core of the enzyme in the cold (*9*). The γ subunit is essential for rapid ATP hydrolysis by CF1 and confers to the enzyme properties not seen in $(\alpha\beta)n$. CF1 deficient in the γ, δ, and ϵ subunits has been prepared (*5*, *10*). The $\alpha\beta$ complex, referred to here as $(\alpha\beta)n$, with n being from 1 to 3, is less stable than $\alpha_3\beta_3\gamma$, binds less nucleotide (*5*), and has low ATPase activity (*5*, *11*). Reconstitution of native γ with $(\alpha\beta)n$ restores high ATPase activity, nucleotide binding, and stability to that of CF1($-\delta\epsilon$) (*5*).

The complexity of the $\alpha_3\beta_3$ structure of CF1 was revealed by electron microscopy to show that the α and β subunits alternate in a pseudohexagonal, apparently symmetric ring (*12*), similar to structures observed in F1 (*13*, *14*). The presence of the small subunits results in overall asymmetry of CF1, because the small, single-copy subunits cannot participate in the apparent 3-fold symmetry of the $\alpha_3\beta_3$ structure. The X-ray crystal structures of mitochondrial F1 from bovine heart (*15*) and rat liver (*16*) have further defined the F1−ATPase structure, although only part of the γ subunit and none of either the δ and ϵ subunits were resolved. The resolution of the crystal structures allows the asymmetry of F1−ATPases to be characterized in terms of the complex contacts between individual α and β subunits and α/β pairs interacting with bound nucleotides and portions of the γ subunit. It also allows predictions to be made concerning

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[1] Abbreviations: CF1, chloroplast coupling factor 1; CF1($-\epsilon$), CF1 lacking ϵ subunit; CF1($-\delta$), CF1 lacking δ subunit; CF1($-\delta\epsilon$), CF1 lacking δ and ϵ subunits; $(\alpha\beta)n$, α and β subunits, with n representing 1−3 copies; F1, coupling factor 1; AMPPNP, adenylyl-β,γ-imidodiphosphate; LY, 4-amino-*N*-[3-(vinylsulfonyl)phenyl]naphthalimide-3,6-disulfonic acid; α-lys_{378}, α subunit lysine at position 378; HPLC, high-performance liquid chromatography; ϵ-C6S, recombinant ϵ subunit with serine replacing cysteine at position 6.

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the interactions between the $\alpha_3\beta_3$ structure and the remaining small subunits.

The positions of the six nucleotide binding sites were also confirmed by the crystal structure. The nucleotides bind at the interfaces of the α and β subunits, approximately halfway down the length of the F1 structure. The properties of the nucleotide binding sites have been and are still being investigated in CF1 (*17*−*19*) and mitochondrial F1 (*20*, *21*). In general, the nucleotide sites on CF1 can be classified into two groups: two loose sites and four tight sites. Tight and loose are operational definitions referring to the ability of the tight sites to retain bound nucleotides through two consecutive centrifuge gel filtration columns. The unique properties of each of the six nucleotide binding sites shows that the enzyme binds nucleotides asymmetrically. This asymmetry is apparent from the nucleotides bound to F1 as revealed from the crystal structures. Even attempting to freeze F1 by symmetrically placing AMPPNP in the noncatalytic α sites results in a crystal structure with overall asymmetry (*15*). The binding of nucleotides to CF1 must have a role in altering the enzyme's structure, either to cause asymmetry or to propagate the already established asymmetry.

The fluorescent probe, Lucifer Yellow vinyl sulfone (LY), covalently binds to a unique site on one of the three α subunits (*22*) of CF1. The rapid binding of LY to one specific position resulted in its use as a site for fluorescence resonance energy-transfer mapping of distances in the intact structure. Many distances have been mapped on CF1 including distances between specific residues (*23*, *24*), distances between bound nucleotides (*25*, *26*), and the distance between specific sites on CF1 and the membrane surface (*27*). Structural mapping was used to show that the LY was bound to a single position, fixed in space by seven independent distance measurements (*22*). The specific α subunit residue that bound LY was determined to be a lysine residue in the α subunit at position 378 (α-lys_{378}) (*28*). Additionally, the binding of LY to CF1 has less than a 10% effect on ATPase activity. Since LY binds rapidly to just one of the three α subunits, the reaction of CF1 with LY may be used as a probe to investigate a cause of asymmetry within the chloroplast ATP synthase structure.

We show here that the reaction of α-lys_{378} of CF1 with LY is unaffected by the bound nucleotide content of the enzyme or by the removal of the δ and ϵ subunits. Removal of the γ subunit stimulated LY incorporation into the α subunits and reconstitution with γ inhibited LY incorporation. Thus, $\gamma-\alpha/\beta$ interactions are responsible at least in part for the asymmetry of the α subunit of CF1.

EXPERIMENTAL PROCEDURES

Purification of CF1. CF1 was purified from market spinach (*29*, *30*). Contaminating ribulose 1,5-bisphosphate carboxylase/oxygenase was removed by immunoaffinity chromatography (*31*). CF1($-\epsilon$), CF1($-\delta$), and CF1($-\delta\epsilon$) were prepared as previously described (*3*, *4*, *29*) and stored in Tris−HCl buffers (pH 8.0) as 50% ammonium sulfate precipitates at 4 °C with 1 mM ATP and 2 mM EDTA. Isolation of ($\alpha\beta$)*n* from CF1($-\delta\epsilon$) was accomplished with minor modifications of the established procedure (*5*). The ($\alpha\beta$)*n* was concentrated and stored with 1 mM $MgCl_2$ and 1 mM ATP at −80 °C.

Nucleotide Loading of CF1. Loading of specific nucleotide binding sites in CF1 was performed according to the established procedure (*19*). Tight Mg^{2+}−ATP sites (N2, N5) were loaded with either 5 mM ATP or 5 mM AMPPNP in the presence of 5 mM $MgCl_2$. Tight ADP (N4) and tight, exchangeable (N1) sites were loaded with 5 mM ADP in the presence of 5 mM EDTA. Excess and loosely bound nucleotides were removed by passage of CF1 through two consecutive, 3-mL Sephadex G-50 centrifuge columns (*32*). Mg^{2+} was bound to ADP tightly bound at the N4 and N1 sites by incubation with 1 mM $MgCl_2$ for 1 h and unbound $MgCl_2$ removed by passing CF1 through Sephadex G-50 gel filtration centrifuge columns (*19*, *33*). The nucleotide content was determined by ion-pairing high-performance liquid chromatography (*29*, *34*) using a Beckman 342 gradient liquid chromatograph with a Chromega chromegabond mc 18/5, 4.6- × 10-cm reversed-phase column. The molecular weight of CF1 is 400 000 (*35*).

Lucifer Yellow Labeling of CF1. Thylakoid membranes were prepared from 500 g of spinach leaves (*31*). One-half of the preparation (70 mg of chlorophyll) was incubated with 50 μM LY for 30 min in the dark while the control received H_2O. CF1 was isolated from both preparations. The stoichiometry of LY labeling was determined spectrophotometrically using the control CF1 to correct for light scattering.

Labeling of soluble CF1 with LY was performed using one of two buffer conditions. Protein (0.5−2 mg/mL) was incubated with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) for 25 °C samples or in 50 mM Bicine−NaOH, 10% glycerol, 1 mM $MgCl_2$, and 1 mM ATP (pH 9.0) for 0 °C samples. Aliquots were taken at the indicated times, and unbound LY was immediately removed by two consecutive gel filtration centrifuge columns, equilibrated with 50 mM Tris−HCl (pH 8.0). The LY fluorescence of each sample was determined on a Shimazu RF-5000 spectrofluorometer and normalized to the protein concentration determined by the Lowry method (*36*). Bound LY was excited at 430 nm, and the fluoresence emission was measured at 520 nm. The increase in the LY fluorescence over the time course was fit by KaleidaGraph version 3 (Abelbeck Software) to the following equation: $F(t) = F_{max}[1 - \exp(-kt)]$, where $F(t)$ is the LY fluorescence at time t and F_{max} is the extent of the fluorescence change. The R values for the data to the fits were between 0.996 and 0.999 for CF1 and between 0.991 and 0.999 for ($\alpha\beta$)*n*. The stoichiometry of labeling was determined spectrophotometrically using an extinction coefficient for LY of 1.22×10^4 $M^{-1}\cdot cm^{-1}$ at 428 nm (*37*).

Analysis of Tryptic Peptides. HPLC separation of tryptic peptides followed a procedure developed by Shapiro (*28*). LY-labeled CF1 (0.5−2 mg/mL) was digested with 0.02 mg/mL trypsin (in 1 mM H_2SO_4) with 5 mM $CaCl_2$ in 50 mM Tris−HCl (pH 8.0) for 2 h at 25 °C. The mixture was boiled for 30 s, and digestion was repeated at 25 °C overnight. Aliquots (100 μL) were analyzed on a Waters 625 LC system with Waters 991 photodiode array detector recording at 428, 278, and 220 nm with a Beckman 157 fluorescence detector and Spectra-Physics 4270 integrator. A linear gradient of 2−45% acetonitrile/0.1% trifluoroacetic acid over 1 h separated peptide fragments on a Waters (Milford, MA) Delta

Pak C_{18}, 3.9- × 150-mm reversed-phase column at a flow rate of 1 mL/min. Integrated absorbance and fluorescence peaks were normalized to the protein concentration. The trypsin fragment containing LY bound to α-lys$_{378}$ had a retention time of 15 min.

Confirmation that the 15-min trypsin fragment contained α-lys$_{378}$ was accomplished by purification of the 15-min fragment (*28*). CF1 (6 mg) labeled with LY either in solution for 50 min or on thylakoid membranes for 30 min was trypsin cleaved as described above. Each trypsin digest was chromatographed on a 1.5- × 10-cm Sephadex G-50 column equilibrated with H_2O. The fluorescent fractions that eluted last were pooled, lyophilized, dissolved in 0.5 mL of H_2O, and rechromotagraphed on a 0.7- × 20-cm Sephadex G-25 column. The fluorescent fractions were pooled, lyophilized, and dissolved in 100 μL of H_2O. The samples were chromatographed on the reversed-phase C_{18} column as described above, and the peptide that eluted at 15 min was collected, lyophilized, dissolved in 100 μL of H_2O, and chromatographed on a Waters Delta Pak C_3, 4.6- × 75-mm reversed-phase column with a linear gradient of 2−7% acetonitrile/0.1% trifluoroacetic acid over 1 h at 1 mL/min. The fluorescent fractions were pooled and lyophilized. Amino acid analysis and matrix-assisted laser desorption ionization mass spectrometry (MALDI) were performed at the Cornell Bioanalytical Facility.

Overexpression of γ and ϵ Subunits. Transformation of BL21/DE3 *Escherichia coli* expression host, induction of expression, purification, solubilization, folding of recombinant ϵ-C6S, and reconstitution with CF1(−ϵ) were performed as previously described (*6*). Transformation of BL21/DE3 (pLysS) *E. coli* expression host, induction of expression, and purification of γ were performed according to the procedures kindly provided by Dr. M. L. Richter, whose laboratory also provided the full length clone for the spinach γ subunit. Thawed inclusion bodies containing approximately 2−5 mg of γ were pelleted, washed with H_2O, and dissolved in 100−200 μL of 25 mM Na_2CO_3, 4 M urea (pH 9.5), on ice. The protein concentration was determined (*38*). Solubilized γ was folded by rapid dilution (1:10) into chilled dilution buffer which contained 10% glycerol, 0.3 M LiCl, 50 mM Na_2CO_3, and 5 mM DTT (pH 9.5). Reconstitution of γ with (αβ)*n* was at a molar ratio of 3 γ to 1 (αβ)*n*, assuming *n* is 3 for (αβ)*n*, with (αβ)*n* diluted to between 0.1 and 0.4 mg/mL in 10% glycerol, 50 mM HEPES−NaOH (pH 6.7), 1 mM $MgCl_2$, 1 mM ATP, and 2 mM DTT. The mixture was incubated for 1 h at 25 °C.

ATPase activities were assayed by incubation of 5−10 μg of protein for 3−10 min at 37 °C in 50 mM Tris−HCl (pH 8.0), 4 mM ATP, 2 mM $MgCl_2$, and 100 mM Na_2SO_3. The amount of Pi produced was determined colorimetrically (*39*).

RESULTS

LY Labeling of CF1. Lucifer Yellow vinyl sulfone is a derivative of naphthylimide that covalently reacts with amino and sulfhydryl groups (*37*). Its fluorescent properties make it ideal for use as an extrinsic probe for protein fluorescence studies in the pH range 5.5−10, where it is insensitive to H^+ concentration. CF1 will rapidly bind LY with the ratio of 1 mol of LY per mol of protein. This covalent interaction

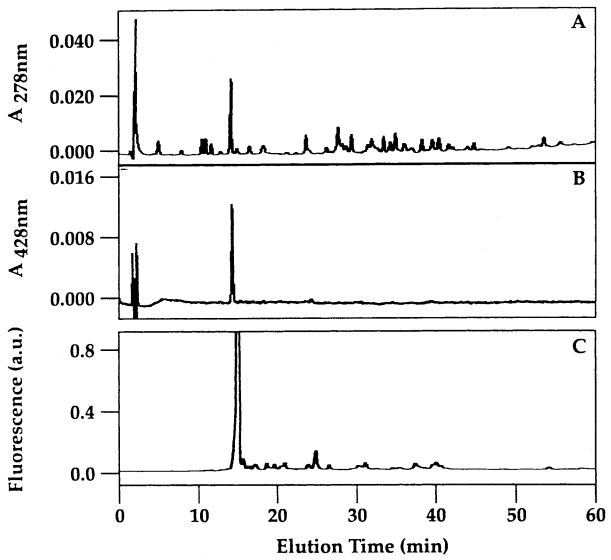

FIGURE 1: High-performance liquid chromatography of trypsin fragments of CF1. CF1 was labeled with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) at 25 °C for 45 min, unbound LY was removed, and LY-labeled CF1 was digested by trypsin to completion as described in the Experimental Procedures section. CF1 (100 μg) was separated on a C_{18} reversed-phase column with a linear gradient of 2−45% acetonitrile/0.1% trifluoroacetic acid over 1 h with the following scans recorded: A, absorbance scan at 278 nm; B, absorbance scan at 428 nm; C, fluorescence scan.

is stable and can be maintained through multiple centrifuge columns or ammonium sulfate precipitation.

The site of LY binding to CF1 was determined to be on the α subunit by fluorescence resonance energy-transfer mapping measurements between LY and probes bound to seven discrete sites on CF1 (*22*) which converged to a single location. Only one of the three α subunits of CF1 reacts with LY. α-lys$_{378}$ was determined to be the α subunit residue that reacts covalently with LY (*28*). The binding of LY to the CF1 α subunit was analyzed by two methods. The fluorescence of the total amount of LY bound to CF1 was measured over a time course of LY incubation with CF1 and normalized to the protein concentration to allow comparison between the samples. HPLC separation of tryptic peptides of LY-labeled CF1 was used to quantify the amount of LY bound specifically to α-lys$_{378}$. Integrated absorbance and fluorescence HPLC peaks were normalized to the protein concentration and used to compare the amount of LY that was bound to the tryptic peptide that contained α-lys$_{378}$ in different preparations.

A representative HPLC experiment of CF1 that was labeled with LY for 45 min and then subjected to complete trypsin digestion is presented (Figure 1). The specificity of attachment of LY to α-lys$_{378}$ is extremely high compared to the number of peptide fragments detected at 220 nm (not shown) or at 278 nm. The peak containing LY−peptide was extremely sharp and narrow when detected either by its absorbance at 428 nm or its fluorescence, further demonstrating the specificity of LY labeling.

Representative time courses of the total fluorescence of LY bound to CF1 and LY that was specifically bound to α-lys$_{378}$ in CF1 are similar (Figure 2). The rapid binding of LY to α-lys$_{378}$ is seen in the first 20 min of LY labeling and levels off as the stoichiometry of LY labeling of CF1, measured spectrophotometrically, approached 1 mol of LY

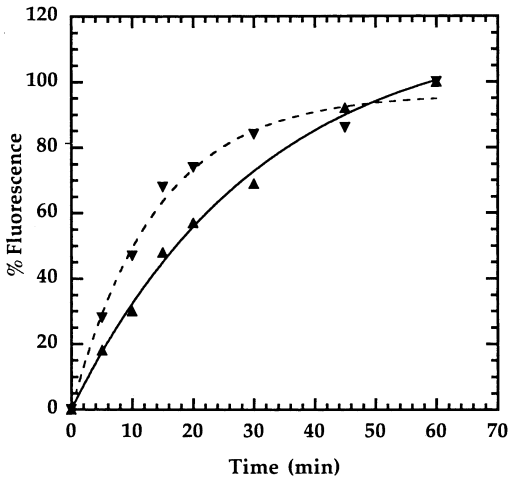

FIGURE 2: Time courses of total LY labeling of CF1 determined by fluorescence (▲) and by the specific labeling of α-lys$_{378}$ (▼). CF1 (2 mg/mL) was incubated with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) at 25 °C. Aliquots were taken at the indicated times, and unbound LY was removed. Fluorescence emission of total LY bound to CF1 was measured at 520 nm and normalized to the protein concentration. The incorporation of LY into α-lys$_{378}$ was determined after trypsin digestion of each aliquot as described in the Experimental Procedures section. The integrated fluorescence of the peptide that elutes at 15 min on a reversed-phase HPLC column was normalized to the protein concentration. The curves shown are a first-order fit to the data. Note that the two experiments shown were not carried out with the same CF1 preparation and are, thus, not strictly comparable. The % fluorescence was determined by dividing the fluorescence at each time point by the fluorescence at 60 min.

bound per mol of CF1 during the time course. The rate constant derived from the curve fit of LY labeling α-lys$_{378}$ was 0.072 min^{-1}, which represents the isolated rate constant of only the LY labeling of the α-lys$_{378}$ residue.

The majority of the LY fluorescence in a trypsin digest of LY-labeled CF1 was present in a peptide that eluted at 15 min from the reversed-phase HPLC column. To reconfirm the identity of the LY-modified residue, the peptide that eluted at 15 min was further purified and analyzed by amino acid analysis and by matrix-assisted laser desorption ionization mass spectrometry. Amino acid analysis showed the presence of four amino acids: glycine, alanine, valine, and lysine. Also present were several unknowns, which were probably fragments of the LY-labeled amino acid. There is only one tryptic fragment in the spinach α subunit that contains one of each of the above residues plus an additional residue that can bind LY: fragment 378−382. The identity of lysine 378 as the residue binding LY was therefore confirmed, because trypsin will not cut after a modified lysine. The calculated mass for the tryptic peptide identified as binding LY is 1040.62 Da, taking into account the mass of LY. Mass spectrophotometry of the purified LY−peptide showed a prominent peak at 1042 Da, within 0.13% of the calculated mass of the peptide (not shown).

LY Labeling of CF1 on Thylakoid Membranes. CF1 attached to CFo in thylakoids was also labeled with LY for 30 min, and the LY-labeled CF1 was purified. The stoichiometry of LY labeling was followed through the purification procedure. After complete removal of ribulose 1,5-bisphosphate carboxylase/oxygenase by immunoaffinity chromatography, 1.16 mol of LY was bound per mol of CF1. To ensure that the same residue was LY labeled as in soluble

Table 1: Influence of Bound Nucleotide Content on the Total LY Labeling of CF1($-\epsilon$) as Determined by LY Fluorescence[a]

bound nucleotide content[b] (mol of CF1($-\epsilon$))$^{-1}$	k (min^{-1})	extent[c]
1.2 mol of ADP	0.028	718
1.1 mol of ADP + Mg^{2+}	0.034	672
1.7 mol of ADP	0.033	657
1.3 mol of ADP, 1.8 mol of ATP	0.027	595
1.4 mol of ADP, 1.1 mol of AMPPNP	0.029	556
1.7 mol of ADP, 1.5 mol of ATP, + 5 mM $MgCl_2$, 5 mM ATP during LY labeling[d]	0.027	631

[a] Rate constants and extents were obtained from a first-order fit to time course data. [b] Samples (2 mg/mL) were labeled with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) at 25 °C. [c] Extents are represented in arbitrary fluorescence units normalized to the protein concentration. [d] In the absence of Mg^{2+}−ATP in the medium, the kinetic values were extent, 629, and k, 0.023 min^{-1}.

Table 2: Comparison of the Extent of Specific Labeling of α-lys$_{378}$ by LY

CF1 sample[a]	n[b]	$A_{428\ nm}$[c] (au/mg of protein)
CF1	7	885 ± 37
CF1($-\epsilon$)	4	909 ± 30
CF1($-\epsilon$) + Mg^{2+}−ATP[d]	3	920 ± 26
CF1($-\epsilon$) + ϵ-C6S	3	902 ± 19

[a] LY was labeled with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) for 50 min at 25 °C. [b] n is the number of independent preparations. [c] Absorbance values are reported ± the standard deviation of the sample mean. [d] CF1($-\epsilon$) has an average of 1.33 mol of ADP and 1.61 mol of ATP bound/mol of protein.

CF1, an aliquot of the labeled CF1 was trypsin digested, and the LY-labeled fragment was purified. The LY-labeled tryptic peptide from CF1 incubated with LY in solution or while bound to the thylakoid membrane had identical resolution times on reversed-phase HPLC columns. Matrix-assisted laser desorption ionization mass spectrometry determined that the experimental mass of the sample was 1041.5 Da, within 0.1% of the calculated mass of the LY tryptic peptide, α 378−382 plus LY.

Effects of Nucleotides on LY Labeling. Characterization of the six nucleotide binding sites on CF1 has led to improved methods for specifically loading the binding sites (*19, 33*). Different combinations of bound nucleotides were investigated for their affects on LY binding to CF1($-\epsilon$) (Table 1). LY incorporation into CF1($-\epsilon$) with different nucleotide contents was determined by total LY fluorescence. The first-order fit of the time course data of LY binding to CF1($-\epsilon$) with the minimal amount of bound ADP has a rate constant of 0.028 min^{-1} and an extent of 718 arbitrary fluorescence units/mg of protein. These values change little as additional ADP is bound to the other tight ADP site or Mg^{2+} is bound to the already tightly bound ADP.

The presence of tightly bound Mg^{2+}−adenosine triphosphates lowered the extent of total LY binding to CF1($-\epsilon$) during the time course (Table 1). The decrease in the extent of LY incorporation is attributed to the slowing of LY incorporation into CF1 at some residue other than α-lys$_{378}$. HPLC determination of the extent of specific incorporation of LY into α-lys$_{378}$ revealed that the LY labeling was unaffected by the presence of tightly bound Mg^{2+}−ATP on CF1($-\epsilon$) (Table 2). Therefore, the presence of tightly bound

Table 3: Influence of Subunit Composition on the Total LY Labeling of CF1 as Determined by LY Fluorescence[a]

CF1 sample[b]	k (min^{-1})	extent[c]
CF1	0.032	419
CF1($-\delta$)	0.029	529
CF1($-\epsilon$)	0.030	587
CF1($-\delta\epsilon$)	0.032	509

[a] Rate constants and extents of labeling were obtained from a first-order fit to the time course data. [b] Samples (2 mg/mL) were labeled with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) at 25 °C. [c] Extent values are represented in arbitrary fluorescence units normalized to the protein concentration.

Mg^{2+}−adenosine triphosphates must reduce the nonspecific LY labeling of a site other than α-lys$_{378}$.

The effect of catalytic turnover of the enzyme on LY labeling was tested by incubation of CF1($-\epsilon$) loaded with tightly bound Mg^{2+}−ATP with LY in the presence of 5 mM $MgCl_2$ and 5 mM ATP at 25 °C. Neither the rate constant nor the extent of labeling by LY was significantly altered by the presence of Mg^{2+}−ATP in the medium (Table 1).

Effects of δ and ϵ Subunits on LY Labeling. CF1 is a complicated structure composed of many subunits that interact. Smaller subunits may be located in a position that would block the access of LY to the α subunit or change the conformation of α subunits, such that the access of LY to α-lys$_{378}$ would be restricted. Depletion of δ, ϵ, or both subunits together provides a means to test for these effects. As the small subunits were removed systematically, a change was observed in the extent of total LY binding determined by fluorescence (Table 3). This increase in the extent of fluorescence of total LY binding was supported by SDS−PAGE of LY labeled samples which showed labeling of the γ subunit, especially when the ϵ subunit was removed (data not shown).

Recombinant ϵ-C6S combines readily with CF1($-\epsilon$), and ϵ-C6S reconstitution with CF1($-\epsilon$) can be tested for by inhibition of ATPase activity (*6*). ϵ reconstitution with CF1($-\epsilon$) blocked the increased extent of total LY labeling observed in CF1($-\epsilon$) during the time course (Figure 3). SDS−PAGE revealed that LY labeling of the γ subunit was reduced when ϵ-C6S was reconstituted with CF1($-\epsilon$) (data not shown) but the α subunit labeling was unaffected.

The extent of incorporation of LY specifically into α-lys$_{378}$ was determined by HPLC for preparations that had been compared by the fluorescence of total bound LY. There was no significant difference in the extent of specific LY labeling of α-lys$_{378}$ in CF1, CF1($-\epsilon$), and CF1($-\epsilon$) reconstituted with ϵ-C6S (Table 2). The minor differences in the extent of total LY labeling within these preparations previously noted can be explained by the labeling of residues other than α-lys$_{378}$; these residues are probably located within the γ subunit.

Effects of the γ Subunit on LY Labeling. The γ subunit, the central unit of CF1, has unique contacts with the inner core of the α/β pseudohexagonal structure. The influence of the γ subunit on each α subunit would be unique and could create a special environment for LY labeling or block the availability of a LY binding site. To test the effects of γ on LY labeling, (αβ)*n* was prepared from CF1($-\delta\epsilon$) (*5*). Adaptations had to be made to the LY-labeling buffer, to allow (αβ)*n* to be LY labeled while remaining active. The buffer contains, in addition to 50 mM Bicine−NaOH at (pH 9.0), 10% glycerol, 1 mM $MgCl_2$, and 1 mM ATP, and the time course of LY labeling was done at 0 °C over 2.5 h. This time course was chosen because analysis of the amount of LY bound to α-lys$_{378}$ revealed that 2 h of labeling CF1 in this buffer at 0 °C resulted in similar levels of labeling as in CF1 incubated for 50 min at 25 °C in just 50 mM Bicine−NaOH (pH 9.0) (refer to Tables 2 and 4). Less than 10% loss of ATPase activity was observed in (αβ)*n* after LY labeling.

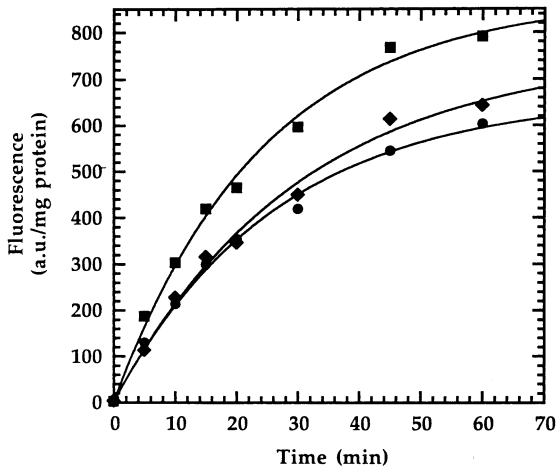

FIGURE 3: Influence of removal and reconstitution of the ϵ subunit on the time course of total LY labeling of CF1. Time course of LY labeling CF1 (●), CF1($-\epsilon$) (■), and CF1($-\epsilon$) + ϵ-C6S (◆). Samples (2 mg/mL) were labeled with 50 μM LY in 50 mM Bicine−NaOH (pH 9.0) at 25 °C. Aliquots were taken at the indicated times, unbound LY was removed, and fluorescence emissions were measured at 520 nm and normalized to the protein concentration.

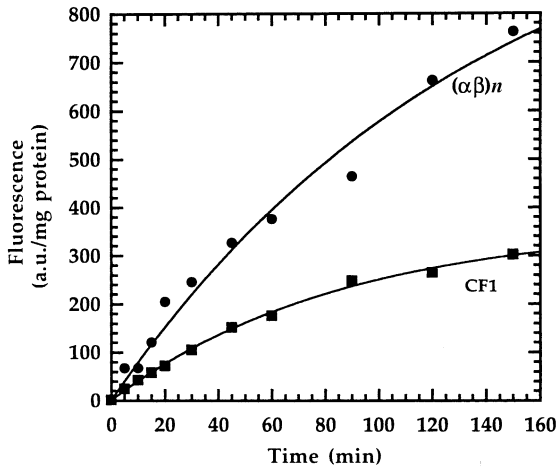

FIGURE 4: Time course of total LY labeling of CF1 (■) and (αβ)*n* (●). Protein samples (1 mg/mL) were incubated with 50 μM LY in 50 mM Bicine−NaOH, 10% glycerol, 1 mM $MgCl_2$, and 1 mM ATP (pH 9.0) at 0 °C. Aliquots were taken at indicated times, unbound LY was removed, and fluorescence emissions were measured at 520 nm and normalized to the protein concentration.

Over the 2.5-h time course, (αβ)*n* bound a greater amount of LY compared to CF1 (Figure 4). The first-order fits to the data gave extent values of 1118 and 363 arbitrary fluorescent units/mg of protein for (αβ)*n* and CF1, respectively, which represents a 3-fold difference. SDS−PAGE of (αβ)*n* and CF1 samples revealed that additional subunits other than the α subunit bound small amounts of LY (not shown), indicating that the overall fluorescence and stoichi-

Table 4: Effects of Depletion and Reconstitution of the γ Subunit on the LY Labeling of α-lys$_{378}$ as Determined by HPLC of Tryptic Peptides

CF1 sample	labeling condition[a]	n[b]	A_{428nm}[c] (au/mg of protein)	n[b]	fluorescence[c] (au/mg of protein)
CF1	0 °C	4	916 ± 80	4	210 992 ± 13 729
CF1($-\delta\epsilon$)	0 °C	6	920 ± 57	6	212 482 ± 8708
($\alpha\beta$)n	0 °C	7	3146 ± 248	5	628 381 ± 39 946
($\alpha\beta$)$n + \gamma$	0 °C	2	953 ± 18	2	253 456 ± 14 791
CF1($-\delta\epsilon$)	25 °C	4	1174 ± 52	5	273 876 ± 13 574
($\alpha\beta$)$n + \gamma$	25 °C	3	1012 ± 68	3	255 133 ± 8670

[a] Labeling conditions were 0 °C in a 50 mM Bicine−NaOH, 10% glycerol, 1 mM $MgCl_2$, and 1 mM ATP (pH 9.0) for 2 h or 25 °C in 50 mM Bicine−NaOH (pH 9.0) for 50 min. [b] n is the number of independent preparations. Absorbance and fluorescence values are reported ± the standard deviation of the sample mean.

ometry measurements were not sufficient to quantitate the amount of LY bound to α-lys$_{378}$.

HPLC separation of tryptic peptide fragments was used to quantitate and compare the LY labeling of ($\alpha\beta$)n and CF1 samples (Table 4). This method eliminates the small amount of nonspecific LY labeling of other subunits and represents the specific labeling of the peptide containing α-lys$_{378}$. The LY bound to α-lys$_{378}$ of ($\alpha\beta$)n was 3 times that of CF1, analyzed by integration of both absorbance at 428 nm and fluorescence HPLC peaks. ($\alpha\beta$)n had an absorbance value of 3159 ± 294 and a fluorescence value of 638 277 ± 33 647, respectively, compared to values of 973 ± 72 and 226 420 ± 7377 for CF1($-\delta\epsilon$) under the 0 °C conditions after being normalized to protein concentrations.

($\alpha\beta$)n was reconstituted with the recombinant γ subunit. Reconstitution was tested by assaying Mg^{2+}−ATPase activity in the presence of sulfite. ($\alpha\beta$)n usually had an activity of 0.2−0.6 μmol of Pi formed/(min·mg). After reconstitution with the γ subunit, the activity was 50−100 times that of ($\alpha\beta$)n. LY-labeled ($\alpha\beta$)n could also reconstitute with γ and return the activity values to within this same high activity range. ($\alpha\beta$)n reconstituted with γ was labeled with LY under the two labeling conditions: 25 or 0 °C. Under both conditions, the amount of LY incorporated into α-lys$_{378}$ in ($\alpha\beta$)n reconstituted with γ was similar to the amount of LY incorporated into α-lys$_{378}$ in CF1($-\delta\epsilon$) under the same conditions (Table 4).

DISCUSSION

The F1−ATPases are large asymmetric structures. Asymmetry within the structure is observed by differences in the three $\alpha\beta$ pairs that make up the structure (*15*, *16*). These differences are the result of interactions between the $\alpha\beta$ pairs and bound nucleotides, the small subunits, and the central γ subunit. One indication of this inherent asymmetry is the covalent and rapid binding of the fluorescent probe Lucifer Yellow vinyl sulfone to only one of the three chemically identical α subunits (*22*). Using the binding of LY to CF1 as a means of probing the structure for the cause of asymmetry, our results clearly demonstrate the importance of the γ subunit in creating an asymmetrical binding site for LY in the pseudohexagonal structure formed by the α and β subunits.

The structural similarity between CF1 and mitochondrial F1 allows the alignment of the α subunit sequences to determine the location of the residue analogous to α-lys$_{378}$ on the crystal structure of rat liver F1 (*16*). Initial analysis indicates that LY would bind on the external face of the α subunit, near the α/β cleft, where the nucleotide binding pocket is located (Amzel, L. M. Personal Communication). The enzyme activity is not affected by bound LY, which indicates that the binding pocket must be large enough to accommodate both molecules during catalysis. Which α subunit binds LY and if there is a nucleotide present at that site have not been determined. Purified CF1 contains approximately 1 mol of tightly bound ADP per mol of CF1. Increasing the tightly bound nucleotide content does not result in additional labeling of α-lys$_{378}$ with LY. Therefore, the presence of tightly bound nucleotides is not sufficient to induce the rapid binding of LY to the remaining two α subunits.

Tightly bound Mg^{2+}−adenosine triphosphates decrease the extent of LY binding to CF1($-\epsilon$) and cause the disappearance of a LY tryptic peptide that has a longer retention time than the LY-labeled α-lys$_{378}$. Tightly bound Mg^{2+}−ATP and Mg^{2+}−AMPPNP stabilize the structure of CF1($-\epsilon$) (*19*, *40*), possibly by tightening the interactions between the α and β subunits. The conformational change in the $\alpha_3\beta_3$ structure upon the binding of Mg^{2+}−adenosine triphosphates may explain the decrease in exposure of potential LY binding sites, other than α-lys$_{378}$. Mg^{2+} has been shown to bind to tightly bound nucleotides on CF1 (*19*, *33*) in amounts equal to those of tightly bound nucleotide (Digel, J. G. Unpublished Results). Fluorescence data show no effect on LY labeling of CF1 either when Mg^{2+} is bound to already tightly bound ADP or when 3 mM $MgCl_2$ is added to the LY labeling buffer, as was previously shown (*22*), indicating that Mg^{2+} has no influence on the binding of LY to CF1.

The δ and ϵ subunits are not involved in the asymmetrical binding of LY to CF1. Chemical cross-linking and labeling experiments in *E. coli* F1 indicate that the ϵ subunit is associated with other subunits including γ (*41*), subunit c (*42*), and α/β pairs through ϵ's C-terminus (reviewed in *43*). The disruption of these associations in CF1 does not result in the relaxation of the α subunits into a symmetrical structure, because a 3-fold increase in the LY labeling of CF1($-\epsilon$) is not observed. From structural modeling of the interactions of *E. coli* ϵ with *E. coli* F1 (*44*, *45*), a substantial portion of the ϵ subunit has been proposed to interact with the γ subunit. Removal of ϵ from CF1 results in an increase in LY labeling of the γ subunit, which can be reversed by reconstitution of ϵ-C6S with CF1($-\epsilon$). This observation provides further evidence for $\gamma-\epsilon$ interaction in CF1 (*29*, *31*). Although there is some evidence for $\alpha-\delta$ interactions (*46*), the δ subunit does not block the reaction of α with LY. Therefore, the δ or ϵ subunit neither blocks access nor promotes the binding of LY to α lys$_{378}$.

The γ subunit has structural importance as the central feature of the ATP synthase. Complexes composed of only the α and β subunits have been found to have lower activity and less stability than the native enzymes (*5*, *11*, *47*−*50*). The binding of three copies of LY to ($\alpha\beta$)n is an indication of the symmetry of this structure's chemically identical α subunits. Introduction of the γ subunit returns the properties to that of CF1 (*5*) and reintroduces the asymmetry of the LY binding. The importance of the γ subunit's interactions with α/β pairs was also revealed in F1 from a thermophilic

bacterium, where the introduction of the γ subunit results in creation of a high-affinity binding site for adenine nucleotides and communication between the catalytic sites in the $\alpha_3\beta_3\gamma$ complex, neither of which is present in the $\alpha_3\beta_3$ complex (*51*).

The asymmetrical binding of LY to CF1 in the soluble form is duplicated while CF1 is bound to the thylakoid membrane. This asymmetric state is maintained after solubilization and purification of CF1. Thus, CFo is not involved in the asymmetric labeling of $(\alpha\beta)n$ with LY.

A question that remains is what happens to the enzyme under catalytic conditions. Fluorescence resonance energy-transfer measurements have shown that incubation of CF1 with Mg^{2+}−ATP or Mg^{2+}−AMPPNP induces switching of the properties of at least two nucleotide binding sites in soluble CF1 (*26*, *52*). These results indicate that the asymmetrical binding of nucleotides is not a permanent feature of the enzyme. This supports the binding change mechanism (*53*, *54*) and the idea that rotation (*15*, *55*, *56*) or large conformational movement (*57*, *58*) of the γ subunit makes the catalytic sites equivalent during the catalytic cycle. Our data show that catalytic turnover results in no additional LY labeling of soluble CF1($-\epsilon$). These data support the idea that the asymmetry of the α subunits is maintained during turnover. The results are, however, inconclusive since alteration in a structure during turnover could relax at a rate too fast to permit increased LY reactivity. Also, it could be that asymmetry, with respect to the labeling of a residue close to the surface of the α subunit, could be maintained during catalysis even though the properties of the catalytic sites may be randomized.

Our results suggest that the γ subunit is responsible for establishing a three-dimensional conformation within $\alpha_3\beta_3$ that is necessary for the asymmetrical binding of LY to CF1. The asymmetrical binding of LY to α-lys_{378} provides a method to determine whether the asymmetry of the α subunit of the enzyme is maintained through activation and catalysis by membrane bound CF1.

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