Influences of Energization and Nucleotide Binding on the Reaction of Lucifer Yellow Vinyl Sulfone with the α Subunits of the Chloroplast ATP Synthase†

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ABSTRACT: The catalytic portion of the chloroplast ATP synthase (CF_1) consists of five different polypeptides in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ and is structurally asymmetric. Asymmetry is readily apparent in the properties of the six nucleotide binding sites and the single-copy, smaller subunits. Asymmetry is also detected in the α subunits by the rapid and covalent binding of Lucifer Yellow vinyl sulfone (LY) to one of the three chemically identical α subunits. The binding of LY to a single α subunit has allowed the investigation of whether asymmetry in the α subunits is a permanent feature of CF_1. The development of an electrochemical proton gradient across illuminated thylakoid membranes and the preincubation of CF_1 in solution with Mg^{2+}-ATP were found to alter the LY distribution such that multiple α subunits were labeled with LY. Illumination of thylakoid membranes doubled the extent of LY labeling, and fluorescence resonance energy transfer measurements indicated that LY was bound to more than one α subunit. Since the change in LY distribution was inhibited by proton ionophores (uncouplers), alteration of α conformation by illumination is a result of the generation of a proton gradient. Preincubation of CF_1 in solution with Mg^{2+}-ATP had no effect on the extent of LY labeling but resulted in multiple α subunits binding LY as determined by fluorescence resonance energy transfer measurements. Adenine nucleotides at substrate level concentrations inhibit the reaction of LY with the α subunits. No increase in LY labeling was observed when thylakoids were illuminated under conditions in which CF_1 was catalytically active.

In photosynthesis, an electrochemical proton gradient is formed upon illumination of thylakoid membranes by proton translocation that is linked to electron transport (*1*, *2*). The movement of protons down the electrochemical gradient, through the chloroplast ATP synthase, provides the energy for the synthesis of ATP from ADP and inorganic phosphate. The chloroplast ATP synthase has two distinct domains, each containing multiple subunits. CF_o is a hydrophobic, membrane-embedded protein complex that contains a proton channel and an attachment site for CF_1[1] to the thylakoid membrane. CF_1, a peripheral membrane protein, performs the catalytic function of the CF_1-CF_o complex. CF_1 contains five subunits in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. The chloroplast ATP synthase (CF_1-CF_o) can be studied as either an intact complex on isolated thylakoid membranes or a soluble protein (CF_1). When CF_1 is isolated from thylakoid membranes it acts as an ATPase.

Much effort has been given to elucidating the overall structure of the ATP synthase and the locations of individual subunits within the complex. Electron microscopy revealed large α and β subunits alternating in a pseudohexagonal ring, with the single-copy γ, δ, and ϵ subunits located underneath or exterior to the $\alpha_3\beta_3$ ring (*3*−*6*). A structural map obtained by fluorescence resonance energy transfer (FRET) provides a framework for determining the location of individual subunits (*7*, *8*). The X-ray crystal structures of mitochondrial F1-ATPases from rat liver (*9*, *10*), bovine heart (*11*), and yeast (*12*) provide details of individual α and β subunits, γ and δ subunits (chloroplast ϵ subunit), 10 c subunits, as well as nucleotide binding regions. Asymmetry of the single-copy F1 subunits creates unique interactions and specialized binding sites with individual α and β subunits. Lucifer Yellow vinyl sulfone (LY) reacts covalently with a single α subunit at position α Lys-378 as confirmed by seven independent FRET distances (*13*, *14*).

Another feature of asymmetry in CF_1 is the differences in the properties of the nucleotide binding sites (*15*−*18*). The distinct properties of individual nucleotide binding sites are created by asymmetrical interactions among the α, β, and γ subunits. Different binding affinities, nucleotide preferences, Mg^{2+} requirements for nucleotide binding, and the ability to exchange with nucleotides in the medium are features of individual nucleotide binding sites. Some of the nucleotide binding sites on CF_1 are considered to be catalytic, while others are thought to play a regulatory or structural role. The different catalytic sites are proposed to participate in an alternating-site mechanism (reviewed in ref *19*), which

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[1] Abbreviations: α Lys-378, α subunit lysine at position 378; AMPPNP, adenylyl β,γ-imidodiphosphate; CF_1, chloroplast coupling factor 1; EM, eosin maleimide; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; LY, 4-amino-*N*-[3-(vinylsulfonyl)phenyl]naphthalimide-3,6-disulfonic acid (Lucifer Yellow); NEM, *N*-ethylmaleimide: PMS, *N*-methylphenazonium methosulfate salt, TNP-ATP (ADP), 2′(3′)-(*O*-trinitrophenyl)adenosine triphosphate (diphosphate).

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involves either two catalytic sites (*20*) or three catalytic sites (*21*). A principle of the alternating-site mechanism is that individual catalytic sites change properties during ATP synthesis or hydrolysis.

Structural asymmetry in the F_1-ATPase is an important issue due to the implications asymmetry has on catalysis. Asymmetry in nucleotide binding sites allows for rapid initiation and termination of enzyme catalysis and multiple-turnover reactions to occur simultaneously. What is not understood is what triggers the structural changes such that the enzyme is released from a single, asymmetric conformation to a dynamic state in which rapid enzyme turnover is accomplished. The trigger for changing the structure of CF_1 has not been determined, but subunit conformational movements, nucleotide binding and release, or both are possible candidates.

The binding of LY can be used to probe the asymmetry of the α subunits in CF_1. We report that the binding of LY to α Lys-378 is influenced by energization of the thylakoid membrane and by nucleotide binding.

MATERIALS AND METHODS

Purification of CF_1. CF_1 was isolated from market spinach by modification of the procedure of Soteropoulos et al. (*22*). QAE chromatography (*23*) and immunoaffinity chromatography (*24*) were used to remove contaminants (*23*). CF_1 was stored as a precipitate in a buffer that contained 2 M $(NH_4)_2SO_4$, borate buffer (75 mM NaCl, 84 mM boric acid, and 25 mM sodium borate, pH 8.4), 1 mM ATP, and 2 mM EDTA.

Lucifer Yellow Labeling of CF_1 in Solution. CF_1 (1−2 mg/mL) was incubated with 50 μM LY in 50 mM Bicine-NaOH (pH 9.0) at 25 °C. After 20 min (or the times indicated in time course experiments) unbound LY was removed by two consecutive Sephadex G-50 centrifuge columns, equilibrated with TN buffer [50 mM Tris-HCl (pH 8.0) and 50 mM NaCl]. The LY fluorescence of the samples from the time course experiments was determined on a Shimadzu RF-5000 spectrophotometer and normalized to protein concentration determined by the Lowry method (*25*). Bound LY was excited at 430 nm, and the fluorescence 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 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 fluorescent change. The R-values for the data to the fits were between 0.996 and 0.999.

Labeling of CF_1 in Thylakoid Membranes with Lucifer Yellow. Thylakoid membranes prepared from 450 g of spinach leaves (*24*) were diluted immediately prior to illumination to 0.1 mg of chlorophyll/mL in 200 mL of a buffer with final concentrations of 50 mM Bicine-NaOH (pH 8.9), 5 mM $MgCl_2$, 50 mM NaCl, 50 μM PMS, and 50 μM LY. The suspension was illuminated with high-intensity (about 2000 W/m^2) light from a quartz−halide bulb with constant stirring at 25 °C (*22*). Thylakoid membranes were also incubated with LY in the dark. After 5 min of incubation, DTT was added to a final concentration of 12.5 mM to react with the remaining LY. Additions to the incubation mixtures are given in legends to figures and tables. Treated membranes were kept on ice and centrifuged at 11300*g* at 4 °C for 30 min. LY-labeled CF_1 was purified from the pelleted membranes.

Stoichiometry of Lucifer Yellow Binding to CF_1. CF_1-LY was desalted by passing the protein through two consecutive Sephadex G-50 centrifuge columns and equilibrated with TN buffer or TNE-8 buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 2 mM EDTA]. The stoichiometry of LY labeling was determined by absorbance with a Beckman DU-70 spectrophotometer and an extinction coefficient for LY of 1.22×10^4 M^{-1} cm^{-1} at 428 nm (*26*). The correction for light scattering was determined with a CF_1 sample that had not been labeled with LY. HPLC analysis of tryptic peptides of LY-labeled CF_1 was used to determine quantitatively the specific labeling of α Lys-378 (*27*).

Incorporation of TNP-ATP (ADP) into Nucleotide Binding Site 1. CF_1-LY was desalted by two consecutive Sephadex G-50 centrifuge columns equilibrated with TNE-8 buffer. Samples labeled with LY in solution or while bound to thylakoid membranes were incubated with TNP-ATP(ADP) for 45 min at 25 °C, and unbound TNP-ATP(ADP) was removed with two consecutive Sephadex G-50 centrifuge columns equilibrated with TNE-8 buffer. Stoichiometry of labeling was determined from the absorbance of TNP-ATP-(ADP) by using an extinction coefficient of 2.51×10^4 M^{-1} cm^{-1} at 418 nm for bound nucleotide (*28*) after correction for light scattering and absorbance of LY at 418 nm. LY-labeled samples contained 0.7−1.0 mol of TNP-ATP(ADP)/mol of LY-labeled protein, the same range as for unlabeled CF_1. The presence of EDTA prevents TNP-ATP from labeling the noncatalytic Mg^{2+}-ATP sites, but nucleotide binding site 1 is specifically labeled with TNP-ADP because it can exchange completely with nucleotides in the medium. These labeling conditions are an improvement over the conditions in which Shapiro and McCarty observed significant energy transfer from LY to TNP-ATP (*29*). In their experiments, the incubation time of the enzyme with TNP-ATP was 120 min (vs 45 min in our experiments) and EDTA was not routinely present during labeling. Fluoresence measurements were made as soon as possible after removal of unbound TNP-ATP in Shapiro's experiments, and some TNP-ADP could have been bound to site 3, from which TNP-ADP may slowly dissociate. The efficiency of energy transfer from LY to TNP-ATP in site 3 is much higher than that from LY to TNP-ATP in site 1 (*13*).

Labeling CF_1 Disulfide Bond Sulfhydryls with Eosin Maleimide. Desalted CF_1 in TNE-8 buffer was incubated at 25 °C with either 5 mM iodoacetic acid for 1 h or 5 mM NEM for 10 min. Unreacted iodoacetic acid or NEM was removed with two consecutive Sephadex G-50 centrifuge columns equilibrated with TNE-8. LY-labeled samples were reduced with 50 mM DTT for 1 h at 25 °C, and passed through two consecutive Sephadex G-50 centrifuge columns equilibrated with TNE-7 [50 mM Tris-HCl (pH 7.0), 50 mM NaCl, and 2 mM EDTA]. The protein was reacted with 10 μM eosin maleimide (EM) (dissolved in *N*,*N*-dimethylformamide at 1 mM) for 5 min at 25 °C and passed through two consecutive Sephadex G-50 centrifuge columns equilibrated with TNE-8. Stoichiometry of labeling was determined from the absorbance of EM by using an extinction coefficient of 9.6×10^4 M^{-1} cm^{-1} at 530 nm (Molecular Probes, Eugene, OR, catalog) after correction for light scatter and LY absorbance. From 1.0 to 1.6 mol of EM/mol of LY-labeled

protein was found.

Fluorescence Resonance Energy Transfer Measurements. Fluorescence resonance energy transfer measurements were performed as described previously (*30*). Fluorescence measurements were performed on an Olis modified SLM Aminco-SPF-500C spectrofluorometer. For all measurements, the donor molecule (LY) was bound to CF_1 prior to the acceptor molecule [either EM or TNP-ATP(ADP)]. For fluorescence resonance energy transfer measurements, the efficiency (E) of energy transfer was determined from

$$E = 1 - F_{DA}/F_D \quad (1)$$

where F_{DA}/F_D is the ratio of the fluorescence emission, in the presence (DA) or absence (D) of the acceptor compound. Corrections were made for acceptor stoichiometry, probe absorbance, light scattering, and differences in protein concentration between samples. CF_1 was diluted to below 1.5 μM in TNE-8 buffer to avoid inner filter effects. Bound LY was excited at 428 nm, and the fluorescence emission was measured at 520 nm. All reported transfer efficiencies were the average of five measurements, obtained on the spectrophotometer in the assay mode, with standard deviations of less than 3% of the average value.

The energy transfer efficiency measurements were used to calculate the distance between specific donor and acceptor sites by using

$$E = \sum_{i=1}^{N_A} (R_0/R_i)^6 \Big/ \left[1 + \sum_{i=1}^{N_A} (R_0/R_i)^6\right] \quad (2)$$

where N_A is the number of acceptors, R_i is the distance between the ith donor−acceptor pair, and R_0 is the distance when the transfer efficiency is 0.5 for a single donor−acceptor pair. Equation 1 assumes that each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of protein. The R_0 value between LY and eosin maleimide is 52.8 Å (*29*) and that between LY and TNP-ATP(ADP) is 34.8 Å (*13*). The assumptions under which the R_0 value is calculated, including the assumption of κ^2 to be $^2/_3$, are discussed elsewhere (*31*).

Nucleotide Loading of CF_1. Loading of specific nucleotide binding sites in CF_1 was performed according to established procedures (*32*). Tight Mg^{2+}-ATP sites (N2, N5) were loaded in the presence of either 5 mM ATP or 5 mM AMPPNP and 1 mM $MgCl_2$, overnight at 25 °C. When N2 and N5 were loaded with Mg^{2+}-ATP, the nucleotide binding sites N4 and N1 bound Mg^{2+}-ADP. Nucleotide content (ADP, ATP, and AMPPNP) was determined by ion-pairing high-performance liquid chromatography (HPLC) (*22*, *33*). The molecular mass of CF_1 is 400 kDa (*34*).

Lucifer Yellow Labeling CF_1 after Incubation with Mg^{2+}-ATP. One-quarter of desalted CF_1 in 50 mM Tris-HCl (pH 8.0) was reequilibrated into 50 mM Bicine-NaOH (pH 8.9) with two consecutive Sephadex G-50 centrifuge columns and set aside (sample A). The remaining CF_1 was incubated with 5 mM ATP and 1 mM $MgCl_2$, overnight at 25 °C. Unbound Mg^{2+}-ATP was removed by two consecutive Sephadex G-50 centrifuge columns. Two-thirds of the protein that had been incubated with Mg^{2+}-ATP (samples B and C) was passed through centrifuge columns equilibrated with 50 mM Bicine-NaOH (pH 8.9) and one-third of the protein (sample D) was passed through centrifuge columns equilibrated with TNE-8. Protein samples A and B were immediately labeled with 50 μM LY for 20 min. Protein samples C and D were allowed to incubate in their buffers overnight at 25 °C, passed through two consecutive Sephadex G-50 columns equilibrated with 50 mM Bicine-NaOH (pH 8.9), and labeled with 50 μM LY for 20 min. LY was removed from all samples by two consecutive Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0) and CF_1-LY samples were stored as 50% ammonium sulfate precipitates at 4 °C, in the presence of 1 mM ATP and 2 mM EDTA. Samples were labeled with TNP-ATP as previously described, and fluorescence resonance energy transfer measurements were made between LY at α Lys-378 and TNP-ATP at nucleotide binding site 1.

Gel Electrophoresis and Assays. The specificity of labeling of CF_1 with LY and EM was analyzed by SDS−polyacrylamide gel electrophoresis on 18% gels (*35*). Chlorophyll concentrations were determined by the method of Arnon (*36*). ATPase activity of CF_1 was measured by Ca^{2+}-ATPase activity in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, and 5 mM $CaCl_2$ at 37 °C for 2−10 min. The amount of P_i produced was determined colorimetrically (*37*). Photophosphorylation with PMS as the mediator was determined as described previously (*22*).

RESULTS

Lucifer Yellow Labeling of CF_1 in Solution or CF_1 Bound to Thylakoid Membranes. Lucifer Yellow vinyl sulfone rapidly and covalently reacts with α Lys-378 (*14*) in one of the three α subunits (*13*) of CF_1 that is in solution or bound to CF_o in thylakoid membranes (*27*). Why Nalin et al. (*13*) failed to observe labeling of CF_1 in thylakoids a number of years ago is not known. In this and a previous study (*27*), labeling of the α subunit of CF_1 in thylakoid membranes was consistently observed.

To determine whether illumination alters the binding of LY to membrane-bound CF_1, isolated thylakoid membranes were illuminated for 5 min in a reaction buffer containing 50 μM LY. The incubation time was limited to 5 min to prevent photodamage to the thylakoid membranes. Thylakoid membranes were also incubated with LY in the dark, as a control, and were illuminated in the presence of uncouplers to ensure that any change in the amount of LY bound to CF_1 during illumination was due to the generation of the electrochemical proton gradient. LY-labeled CF_1 was purified from the treated thylakoid membranes. LY binding affects neither photophosphorylation activity nor the ATPase activity of CF_1 (data not shown).

The amount of LY bound to α Lys-378 was determined from the fluorescence and absorbance of the tryptic peptide that contained the α Lys-378 residue modified with LY (*27*). As is the case for CF_1 in solution, LY reacts quite specifically with α Lys-378 in each of the CF_1 samples isolated from thylakoid membranes. CF_1 from thylakoid membranes illuminated in the presence of LY bound nearly twice as much LY to the α Lys-378 residue as that from thylakoid membranes incubated in the dark or illuminated in the presence of uncouplers (Table 1). Approximately 0.6 mol of LY was bound/mol of CF_1 isolated from thylakoid membranes incubated with LY in the dark or illuminated in

Table 1: Comparison of LY Labeling of α Lys-378 in Dark, Illuminated, and Uncoupled Thylakoid Membranes[a]

thylakoid treatment	*n*	A_{428nm} (AU/mg of protein)	fluorescence (AU/mg protein)
dark	4	211 ± 29	54 835 ± 11 894
light	6	424 ± 25	98 509 ± 11 063
uncoupled	2	253 ± 47	57 286 ± 10 673

[a] Thylakoids were incubated with 50 μM LY in reaction buffer for 5 min in the dark, light, and light plus uncouplers (5 mM NH_4Cl and 2 μM gramicidin). CF_1 was purified and trypsin-digested to completion. Peptides (~200 μg) were separated by reversed-phase HPLC and absorbance and fluorescence of the peptide that eluted at 15 min were integrated. *n* is the number of independent preparations. Absorbance and fluorescence values are reported ± the standard deviation of the mean.

the presence of uncouplers, and 1.1 mol of LY was bound/mol of CF_1 isolated from thylakoid membranes incubated with LY during illumination.

FRET Measurements of CF_1 Labeled with LY. The doubling of the extent of LY incorporation into α Lys-378 by the formation of a electrochemical proton gradient across the thylakoid membranes could result from either enhancement of the rate of incorporation into one α subunit or incorporation into more than one α subunit. FRET was used to determine if LY bound to CF_1 was located at a single α Lys-378 site or at multiple α Lys-378 sites. FRET measurements were made between LY at α Lys-378 and TNP-ATP bound to nucleotide binding site 1 and between LY at α Lys-378 and eosin maleimide (EM) bound to the reduced γ disulfide.

CF_1-LY was labeled with TNP-ATP in the presence of EDTA to ensure that TNP-ATP bound specifically to nucleotide binding site 1 (*31*). Site 1 is the only nucleotide binding site that can rapidly exchange tightly bound ADP for TNP-ATP in the medium, in the absence of Mg^{2+}, therefore ensuring specificity of bound TNP-ATP (*38*).

The extent of TNP-ATP incorporation into CF_1 labeled with LY was close to 1 mol of TNP-ATP bound/mol of CF_1 regardless of the method of LY incorporation into CF_1. The established FRET distance between LY at α Lys-378 and TNP-ATP in site 1 is greater than 57 Å (*13*). The low energy transfer efficiency coupled with the R_0 value for this donor/acceptor pair precludes the calculation of a precise distance between LY at α Lys-378 and TNP-ATP at nucleotide binding site 1. Even though an exact distance cannot be determined, a change from the established distance would suggest that the additional bound LY was at multiple α Lys-378 sites.

Fluorescence emission scans were taken for CF_1 labeled with LY in solution and CF_1 labeled with LY when bound to thylakoid membranes, in the presence and absence of TNP-ATP (Figure 1). The difference in the emission of LY in the presence and absence of TNP-ATP under each LY labeling condition represents resonance energy transfer between LY and TNP-ATP. The addition of sodium dodecyl sulfate to a final concentration of 0.1% abolished the quenching of LY fluorescence seen in the CF_1 sample from thylakoids illuminated in the presence of LY. As shown in Table 2, CF_1 labeled with LY in solution has a transfer efficiency of 0.037 between LY and TNP-ATP, in agreement with a previous measurement (*13*). CF_1 samples labeled with LY in illuminated membranes had an average transfer efficiency of

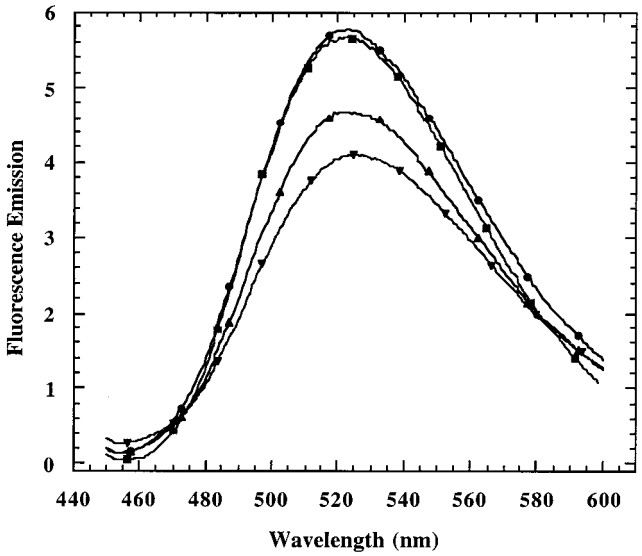

FIGURE 1: Fluorescence scans of CF_1 labeled with LY in solution and in illuminated thylakoid membranes ± TNP-ATP. Samples were labeled with LY and TNP-ATP as described under Materials and Methods. CF_1-LY (●), CF_1-LY-TNP-ATP (■) labeled in solution, CF_1-LY (▲), and CF_1-LY-TNP-ATP (▼) from illuminated thylakoids were excited at 428 nm and emission scans were taken from 450 to 600 nm. The fluorescence value at 520 nm was used in fluorescence resonance energy transfer calculations. Symbols are used to represent the different scans and are only a fraction of the data points taken.

Table 2: Fluorescence Resonance Energy Transfer Measurements in CF_1[a]

condition of LY labeling	D/A pair	*n*	*E*
in solution	LY/TNP-ATP	9	0.037 ± 0.024
thylakoids in dark	LY/TNP-ATP	2	0.061
illuminated thylakoids	LY/TNP-ATP	11	0.163 ± 0.034
uncoupled thylakoids	LY/TNP-ATP	3	0.060
in solution	LY/EM	2	0.210
illuminated thylakoids	LY/EM	2	0.200

[a] CF_1 was labeled with 50 μM LY in solution for 15 min in dark, illuminated, or uncoupled (5 mM NH_4Cl and 2 μM gramicidin) thylakoids for 5 min. CF_1-LY samples were labeled with TNP-ATP at nucleotide binding site 1 or with EM at the reduced γ disulfide. *n* is the number of independent measurements. *E* values are the efficiency of energy transfer, which are reported ± the standard deviation of the sample mean.

0.163 between LY and TNP-ATP. The increase in energy transfer from LY to TNP-ATP in CF_1 from thylakoids illuminated in the presence of LY suggests that LY was incorporated into more than one α subunit under these conditions. CF_1 samples labeled with LY in dark and uncoupled thylakoid membranes had transfer efficiencies of 0.061 and 0.060 between LY and TNP-ATP, respectively, slightly more than that determined for CF_1 labeled with LY in solution. This slight difference may result from small amounts of LY binding to another α Lys-378 site.

FRET efficiencies between LY at α Lys-378 and EM bound to the cysteine residues (Cys-199 and Cys-205) that form the disulfide bond in the γ subunit under oxidizing conditions were also determined. To ensure specificity of labeling with EM, all other exposed cysteine residues in CF_1 were blocked with either NEM or iodoacetic acid, and following reduction of the γ disulfide, EM labeled only the dithiols. The presence of LY bound to CF_1 prior to labeling

Table 3: Effect of Low Concentration of Nucleotides on the LY Labeling of α Lys-378 on Dark, Illuminated, and Uncoupled Thylakoid Membranes[a]

thylakoid treatment	nucleotide addition: none	10 μM ATP	10 μM AMP-PNP	10 μMATP + 3 mM Na_2HAsO_4
dark	54 835	47 238	52 942	58 309
light	98 509	81 474	82 295	73 211
uncoupled	57 286	54 197	58 962	57 926

[a] Thylakoids were incubated with 50 μM LY for 5 min in the dark, light, or light plus uncouplers (5 mM NH_4Cl and 2 μM gramicidin). CF_1 was purified and trypsin-digested to completion. Peptides were separated by reversed-phase HPLC and fluorescence of the major LY-containing peptide was intergrated. Values represent LY fluorescence of the tryptic peptide containing α Lys-378 normalized to protein amount. Buffer-only values are repeated from Table 2.

with EM and the method of labeling CF_1 with LY have no effect on the labeling stoichiometry of EM. The efficiency of energy transfer between LY at α Lys-378 and EM at the reduced disulfide cysteines was previously shown to be 0.210 (*7*). Energy transfer from LY to EM in CF_1 labeled with LY in solution was the same as that in CF_1 labeled with LY in illuminated thylakoids (Table 2).

Effect of Low Concentrations of Nucleotides on LY Labeling. Low concentrations of adenine nucleotides partially protected the γ Cys-89 residue in CF_1 from labeling with NEM during illumination (*39*). Thylakoid membranes were incubated with LY in the presence of 10 μM ATP or 10 μM AMPPNP in the dark, light, or light in the presence of uncouplers (Table 3). CF_1-LY isolated from illuminated samples in the presence of ATP and AMPPNP contained significantly less LY at α Lys-378 than CF_1 isolated from thylakoid membranes illuminated in only the reaction buffer. The presence of low concentrations of nucleotides had smaller, less reproducible effects on the extent of LY incorporation into CF_1 when the incubations were carried out in the dark or in the light plus uncouplers. In the presence of ATP, arsenate, a phosphate analogue, had no effect on the LY labeling of CF_1 from thylakoid membranes incubated in the dark or light plus uncouplers but significantly decreased the labeling in the light (Table 3).

LY Labeling of CF_1 *in Thylakoid Membranes under Catalytic Conditions.* If, as suggested by the binding change mechanism, all three potentially catalytic sites contribute to turnover, the α subunits should alternate among three asymmetric states. The extent of LY incorporation into α Lys-378 should, thus, be increased by catalytic turnover. Thylakoid membranes were incubated with ADP and arsenate during the LY incubation. The arsenate replaced inorganic phosphate and acted as a regenerating system. ADP and arsenate form an unstable bond that spontaneously cleaves after release of the arsenyl-ADP compound from the enzyme (*40*). ADP and arsenate inhibited labeling under all conditions (Table 4). A similar decrease in the amount of LY bound to α Lys-378 by ADP and arsenate was found when the enzyme was inhibited with tentoxin prior to nucleotide or LY addition. Preincubation of thylakoid membranes with tentoxin inhibits ATP synthesis and hydrolysis, essentially trapping the enzyme in a single asymmetric conformation (*41*).

Effects of Medium Nucleotides on LY Labeling of CF_1. Nucleotides tightly bound to CF_1 were found (*27*) to have

Table 4: LY Labeling of α Lys-378 in Thylakoid Membranes under Catalytic and Tentoxin-Inhibited Conditions[a]

thylakoid treatment	condition: catalytic	catalytic + tentoxin
dark	38 142	37 461
light	42 452	37 747
uncoupled	40 299	37 256

[a] Thylakoids were incubated with 50 μM LY for 5 min in 1 mM ADP and 3 mM Na_2HAsO_4 (catalytic condition). The tentoxin concentration was 5 μM. Uncouplers were 5 mM NH_4Cl and 2 μM gramicidin. CF_1 was purified and trypsin-digested to completion. Peptides (~200 μg) were separated by reversed-phase HPLC. Values represent integrated LY fluorescence of the tryptic peptide containing α Lys-378 normalized to protein amount.

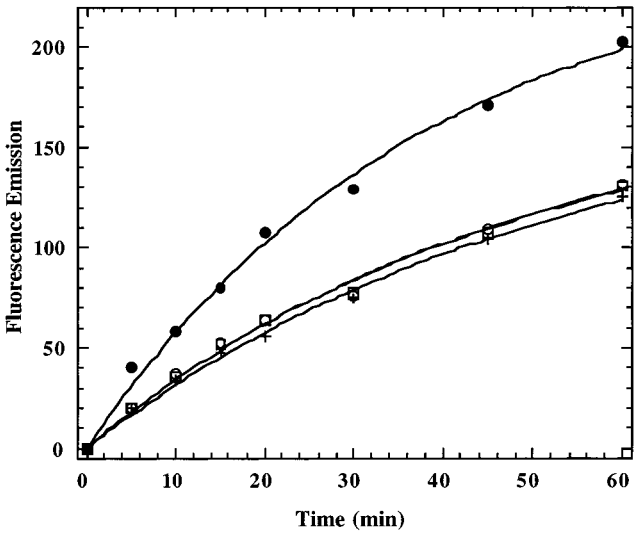

FIGURE 2: Effect of nucleotides in the medium on the LY labeling of CF_1. CF_1 samples (2 mg/mL) were labeled in solution with 50 μM LY in 50 mM Bicine-NaOH (pH 8.9) at 25 °C. The fluorescence of the total LY bound to CF_1 was measured over a time course. CF_1 samples, alone (●) and in the presence of 1 mM ADP (pH 7.0) (+), 1 mM ADP (pH 7.0) and 3 mM Na_2HAsO_4 (○), and 1 mM ATP (pH 7.0) (□) were incubated with LY. At the times indicated an aliquot was taken, unbound LY was removed, and total fluorescence emission at 520 nm was measured and normalized to protein concentration.

no effect on the labeling of CF_1 in solution with LY. The effect of nucleotides in the medium was not thoroughly addressed in that study, although it was found that the LY labeling of CF_1 that contained 2 mol of bound Mg^{2+}-ATP and 1 mol of ADP/mol of enzyme was not affected by Mg^{2+}-ATP in the medium. The observed decrease in the binding of LY to CF_1 in thylakoid membranes in the presence of low concentrations of nucleotides and under catalytic conditions required the investigation of the influence of nucleotides in the medium on the binding of LY to CF_1. CF_1 in solution was labeled with LY in the presence of ATP, ADP, or ADP and arsenate. A decrease in the amount of total LY that bound to CF_1 was observed in all samples that included nucleotides (Figure 2). The specific binding of LY to α Lys-378 was also decreased to a similar extent by nucleotides (data not shown). Thus, soluble and membrane-bound CF_1 respond similarly to the presence of nucleotides in the medium with respect to LY labeling.

Direct interactions between LY and ADP are not the cause in the decrease of the labeling of CF_1 by ADP. The LY labeling of catalase, which contains no nucleotide binding

sites or nucleotide binding site consensus sequences, is not affected by ADP (data not shown).

Nucleotides in the medium inhibit the reaction of LY with α Lys-378 of CF_1 in thylakoid membranes. It was also noticed that the efficiency of energy transfer from LY to TNP-ADP in nucleotide binding site 1 was significantly higher in CF_1 labeled with LY in the presence of Mg^{2+}-nucleotides than in their absence. Efficiency values ranged from 0.11 to 0.16 for CF_1 labeled in the presence of Mg^{2+}-nucleotides in contrast to the 0.04 value obtained in the absence of nucleotide.

Shapiro and McCarty (*29*, *42*) determined that nucleotide binding sites 1 and 3 switched properties during Mg^{2+}-ATP binding. N1 binds nucleotides tightly but exchanges with nucleotides in the medium, and N3 is a dissociable site with a dissociation constant in the micromolar range. After exposure of CF_1-LY to Mg^{2+}-nucleoside triphosphates, the efficiency of energy transfer from LY to the bound TNP-ADP increased significantly, indicating that some of the tightly bound TNP-ADP was located in a position much closer to the LY than is normally observed.

An approach similar to that used in the site-switching experiments was adopted to test the effects of nucleotide binding on the reaction of LY with the α subunits. CF_1 was incubated with or without Mg^{2+}-ATP before being labeled with LY. Unbound nucleotide and Mg^{2+} were removed. Samples were either labeled with LY immediately or allowed to incubate overnight in LY labeling buffer or in a buffer containing EDTA, prior to the addition of LY. All samples were precipitated with ammonium sulfate (2 M) in the presence of EDTA. This storage condition removes all tightly bound Mg^{2+}-ATP by chelating bound Mg^{2+}, causing complete dissociation of tightly bound Mg^{2+}-ATP from the enzyme. TNP-ATP was then specifically bound to N1. FRET from LY to TNP-ATP for the different preparations was determined.

CF_1 that contained tightly bound Mg^{2+}-ATP and ADP during LY labeling showed greater energy transfer from LY to TNP-ATP than CF_1 that contained only tightly bound ADP (Table 5). When CF_1 was labeled with LY immediately after nucleotide removal, the efficiency of energy transfer was 0.12. A similar transfer efficiency was found for CF_1 after incubation in the LY labeling buffer. At the time of LY labeling, both of these CF_1 samples contained bound ATP as well as ADP. The enhancement in energy transfer suggests that the binding or presence of Mg^{2+}-ATP changes the configuration of the enzyme such that some of the LY is located in a position very close to the TNP-ATP at nucleotide binding site 1. If, however, CF_1 was incubated in a buffer containing EDTA prior to incubation with LY, the efficiency of energy transfer between LY and TNP-ATP fell to 0.06, indicating that most of the LY is located on an α Lys-378 that is far from nucleotide binding site 1.

Table 5: Influence of Incubation with Mg^{2+}-ATP on the Labeling of CF_1 in Solution by LY[a]

sample	nucleotide content (mol of nucleotide/mol of CF_1)	*E* LY−TNP-ATP
A	1.54 mol of ADP	0.06
B	1.60 mol of ADP 2.19 mol of ATP	0.12
C	1.73 mol of ADP 1.61 mol of ATP	0.13
D	1.88 mol of ADP	0.06

[a] CF_1 was divided into four parts, three of which (B, C, and D) were loaded with Mg^{2+}-ATP during an overnight incubation in 5 mM ATP and 1 mM $MgCl_2$, in TN buffer. Samples had (A) no Mg^{2+}-ATP preincubation and were labeled with LY immediately; (B) Mg^{2+}-ATP preincubation and were labeled with LY immediately; (C) Mg^{2+}-ATP preincubation and postnucleotide incubation with 50 mM Bicine-NaOH (pH 8.9); or (D) Mg^{2+}-ATP preincubation and postincubation in TNE-8. Aliquots were removed for nucleotide analysis prior to LY labeling and nucleotide content was determined as described under Materials and Methods. Samples were labeled in 50 μM LY and 50 mM Bicine-NaOH (pH 8.9) for 20 min, and excess LY was removed by centrifuge columns. All samples were precipitated by 50% saturation with ammonium sulfate, 1 mM ATP, and 2 mM EDTA to remove tightly bound Mg^{2+}-ATP. Samples were labeled with TNP-ATP, and FRET measurements were determined from LY at α Lys-378 to TNP-ATP bound to nucleotide binding site 1.

DISCUSSION

The reaction of LY to a single α subunit in CF_1 is well established by stoichiometry measurements and seven FRET measurements (*13*). The extent of LY labeling of α Lys-378 of CF_1 in thylakoids is doubled by formation of the electrochemical proton potential. More compelling evidence that the potential caused more than one α Lys-378 to react is the increase in the efficiency of FRET from LY to TNP-ADP, bound to nucleotide binding site 1, in samples incubated with LY in the light. Exposure of CF_1 in solution to Mg^{2+}-ATP prior to LY labeling caused a similar increase in energy transfer from LY to TNP-ADP. The conclusion that more of the incorporated LY is closer to nucleotide binding site 1 in the CF_1 from illuminated thylakoids or incubated with Mg^{2+}-ATP is inescapable. More than one α Lys-378 must be labeled under these conditions.

Removal of the γ subunit of CF_1 results in labeling of all α subunits with LY (*27*), eliminates nucleotide communication between catalytic nucleotide binding sites (*43*), and dramatically changes the properties of the enzyme (*44*). In view of the role of the γ subunit in inducing asymmetry in the α subunits and knowledge that removal of the γ subunit releases CF_1 from asymmetry, conformational changes in γ induced by illumination could be responsible for both the increase in binding of LY and its reaction with multiple α subunits.

In addition to providing the driving force for ATP synthesis, the electrochemical proton gradient activates the ATP synthase in at least three ways. Changes in the structure of CF_1 are induced by the proton potential that weaken interactions between γ and ε subunits, cause the dissociation of inhibitory ADP, and expose the γ disulfide to reduction. Evidence for CF_1 subunit movement in energized thylakoid membranes includes the labeling of γ Cys-89 by NEM (*45*), the labeling of ε Lys-109 (*46*), the accessibility of the γ subunit to trypsin cleavage and reduction (*47*−*49*), and the exposure of the ε subunit to removal by anti-ε antibodies (*50*).

The properties of the increased reactivity of α Lys-378 to LY by illumination are similar to those of the enhanced reactivity of γ Cys-89 to NEM by illumination (*45*). Both reactions are prevented by uncouplers and are decreased by the presence of low concentrations of nucleotides. In both cases, the effect of nucleotide was augmented by arsenate. Although these similarities could be coincidental, they raise

the possibility that the increases in α and γ subunits' reactivity may result from a common structural change induced by the proton potential.

One of the objectives of using LY as a probe of α-subunit asymmetry was to explore the conformation of the α subunits under catalytic turnover conditions. The γ subunit has been proposed to rotate or switch conformations during the catalytic cycle, initiating changes in the nucleotide binding sites necessary to bind reactants, form product, and release product (*51*). LY reactivity should be a sensitive probe of the conformation of the α subunits during catalysis, but the fact that nucleotides at the concentrations necessary for catalysis partially block the binding of LY to CF_1 complicates interpretation of the data. It is noteworthy that turnover did not increase the amount of LY incorporated into CF_1 in illuminated thylakoids as would be predicted if the α subunits were to become equivalent during the catalytic cycle. During the 5 min of illumination, it may be calculated that the CF_1 turned over about 10^5 times.

Nucleotides in the medium decreased LY binding to CF_1. As prepared, CF_1 contains 1.2−1.6 mol of ADP/mol of CF_1, leaving two tight, regulatory (or structural) sites and two dissociable sites available to bind Mg^{2+}-nucleotides. Since nucleotides bound to tight, regulatory sites had no influence on the amount of LY that bound to α Lys-378 (*27*), it is likely that nucleotides in the medium exert their effects by binding to one or both of the dissociable sites. Nucleotides binding to the dissociable sites could either directly block the LY binding site or change the conformation of the structure such that the binding of LY to α Lys-378 is impaired. The analogous region to α Lys-378 in the mitochondrial F_1-ATPase structure is very close to the nucleotide bindings sites (*10*, *11*).

Preloading CF_1 with tightly bound Mg^{2+}-nucleotides was previously shown (*27*) not to affect the amount of LY that bound to α Lys-378, but the distribution of the bound LY was not investigated. FRET between previously bound LY and TNP-ADP at site 1 was used to show that two nucleotide-binding sites switched properties upon the binding of Mg^{2+}-nucleoside triphosphate (*29*). The observation that the exposure of CF_1 to Mg^{2+}-ATP causes LY to react with more than one α Lys-378 confirms the idea that nucleotide-binding initiates switching of nucleotide properties between at least two nucleotide-binding sites.

Site switching may explain why LY binds to multiple α subunits in CF_1 bound to dark or uncoupled thylakoid membranes in the presence of low concentrations of nucleotides. CF_1 isolated from these membranes bound an equivalent amount of LY as thylakoid membranes labeled with LY in the absence of nucleotides, but the FRET measurements indicated that the LY was distributed between multiple α subunits if nucleotides were present during labeling. Mg^{2+}-nucleoside triphosphates seem to be able to bind and initiate site switching in membrane-bound, nonenergized CF_1, suggesting that CF_1 in nonenergized membranes may have an empty dissociable binding site for Mg^{2+}-ATP, similar to CF_1 in solution.

The nucleotide binding sites proposed to participate in nucleotide site-switching were a dissociable binding site, N3, and a tight, exchangeable binding site, N1 (*29*). We predict that LY binds to the α Lys-378 residues close to these two nucleotide binding sites, after preincubation with Mg^{2+}-ATP.

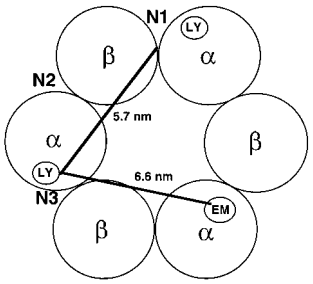

FIGURE 3: Working model of the chloroplast ATP synthase. Top view of the α and β subunits of CF_1 with proposed locations of the LY binding sites at α Lys-378, three of the six nucleotide binding sites, N1−N3, and the EM binding site at the γ disulfide. Distances between nucleotide binding sites are based on fluorescence resonance energy transfer measurements in CF_1 (*7*, *16*). Nucleotide assignments are based on fluorescence resonance energy transfer distances in CF_1 and distances in the bovine MF1 X-ray crystal structure (*7*, *11*) (personal communication, W. Frasch).

We would also predict that these are the two sites labeled with LY in light-energized, membrane-bound CF_1, assuming the simplest model of two, equally labeled α subunits. A new model of the fluorescence resonance energy transfer map has been constructed (Figure 3). Assignments of nucleotide binding sites were based on a comparison between the nucleotides bound to the X-ray crystal structure of bovine mitochondrial F_1-ATPase and the nucleotide characteristics and FRET distances among nucleotide binding sites in CF_1 (personal communication, W. Frasch). The α Lys-378 residue that normally labels with LY is located next to N3 and the new α Lys-378 residue that labels only under light-energized, membrane-bound conditions or after preincubation with Mg^{2+}-ATP is located next to N1. The assignments satisfy the distances between LY and TNP-ATP at nucleotide binding site 1 and LY and EM at the disulfide bond and agree with well-established nucleotide binding site characteristics.

The interactions among α and γ subunits give rise to asymmetry of the α subunits. The effects of the electrochemical proton potential and of medium nucleotides on LY labeling of the α subunit(s) may be induced by structural changes in the γ subunit.

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