ADP Binding Induces Long-Distance Structural Changes in the β Polypeptide of the Chloroplast ATP Synthase†

Denise A. Mills, Steve A. Seibold, Thomas C. Squier, and Mark L. Richter*

Department of Biochemistry, The University of Kansas, Lawrence, Kansas 66045

Received October 27, 1994; Revised Manuscript Received February 24, 1995®

ABSTRACT: Binding of ADP to the β polypeptide isolated from the catalytic F_1 portion (CF_1) of the chloroplast ATP synthase caused an increase of 10−20% in the steady state fluorescence intensity of fluorescent maleimides attached to the cysteine residue at position 63. Fluorescence lifetime distributions indicated that the β polypeptide switched between two conformational states depending on the presence or absence of bound ADP. The fluorescence enhancement induced by ADP binding allowed a direct calculation of the dissociation constant for ADP of 0.7 μM. ATP did not cause a fluorescence enhancement but competed with ADP for binding to the same site. An apparent dissociation constant of 2 μM was obtained for ATP binding. Fluorescence resonance energy transfer experiments indicated that Cys63 is 42 Å away from the nucleotide binding site on the β polypeptide, confirming a previous measurement [(Colvert, K. K., Mills, D. A., Richter, M. L. (1992) *Biochemistry 31*, 3930−3935]. Frequency domain fluorescence anisotropy measurements indicated that the β polypeptide has an irregular, elongated shape which is in good agreement with the conformation found in the crystal structure of the beef heart mitochondrial F_1 enzyme [Abrahams, J. P., Leslie, A. G. W., Lutter, R., & Walker, J. E. (1994) *Nature 370*, 621−628]. The rotational correlation time did not change significantly upon ADP binding, indicating that ADP did not induce a large change in the overall shape of the β polypeptide. The results show that the nucleotide binding domain and the N-terminal domain of the β polypeptide communicate with each other over a significant distance via conformational changes. This supports several other recent findings which have indicated that the N-terminal region of the β polypeptide forms a site of contact with the α polypeptide and that this contact site is important for cooperative exchange of information between nucleotide binding sites during catalysis by CF_1.

The chloroplast coupling factor 1 (CF_1)[1] utilizes the energy of a transmembrane proton gradient to catalyze ATP synthesis. CF_1 is comprised of five different polypeptides designated α−ε in order of decreasing molecular weight, with a polypeptide stoichiometry of 3α, 3β, 1γ, 1δ, and 1ε (McCarty & Moroney, 1985). The two larger polypeptides, α and β, alternate in a hexameric structure, while the three smaller single-copy polypeptides bind asymmetrically to the hexamer (Boekema & Bottcher, 1992; Abrahams et al, 1994). The minimum structure that has so far been shown necessary for normal catalysis (ATP hydrolysis) is $\alpha_3\beta_3\gamma$ (Hu et al., 1993). The nucleotide binding/catalytic sites are located on the β and α polypeptides, whereas the γ polypeptide regulates the activity of the enzyme [reviewed by Nalin and Nelson (1987)].

As many as six nucleotide binding sites with markedly different properties have been identified on CF_1 (Bruist & Hammes, 1981; Cerione & Hammes, 1982; Snyder & Hammes, 1984; Leckband & Hammes, 1987; Xue et al., 1987; Shapiro et al., 1991). Some sites are considered to be catalytically competent, while some may assume a regulatory role. Apparent asymmetry among putative catalytic sites forms the basis for the *alternating sites* hypothesis [reviewed by Boyer (1993)] which attempts to explain the well-known cooperativity in nucleotide binding and catalysis by the ATP synthases. Shapiro and McCarty (1988, 1990) provided some direct evidence for this model when they showed that at least two of the three nucleotide binding sites on CF_1 switch between *tight* and *loose* nucleotide binding during catalytic turnover.

Duncan et al. (1986) proposed that the transfer of information between the different nucleotide binding sites on the F_1 enzymes is propagated via conformational interactions between the different α and β polypeptides. Indeed, several studies (Ohta et al., 1980; Garboczi et al., 1988; Harris & Nadanaciva, 1989) have indicated that the isolated F_1 β polypeptide undergoes significant conformational change upon nucleotide binding. Colvert et al. (1992) showed that the single sulfhydryl residue of the CF_1 β polypeptide located at position 63 in the amino acid sequence is normally buried within the enzyme complex but becomes accessible to labeling reagents in the medium upon isolation of β from other CF_1 polypeptides. This observation suggested that Cys63 is located at or close to an α−β interface, since there are three copies of each of these polypeptides and only single copies of the three smaller polypeptides which are asymmetrically distributed within the enzyme complex (Boekema & Bottcher, 1992). β Cys63 was labeled with fluorescent

† This work was supported by grants from the National Science Foundation (BIR-9116212), the United States Department of Agriculture (93-37306-9633), the National Institutes of Health (GM46837), and the University of Kansas (GRF 3448).

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* May 1, 1995.

[1] Abbreviations: CF_1, chloroplast coupling factor 1; TNP-ATP and TNP-ADP, 2′,3′-*O*-(2,4,6-trinitrophenyl) derivatives of ATP and ADP; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl]ethyl)glycine; Tris, tris-(hydroxymethyl) aminomethane; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl] maleimide; PM, *N*-(1-pyrenyl)maleimide.

0006-2960/95/0434-6100$09.00/0

maleimide derivatives, and a distance of 42 Å between this site and the fluorescent nucleotide analog (trinitrophenyl)-ADP (TNP-ADP), located at the nucleotide binding site on β (Mills & Richter, 1990, 1991), was calculated from data obtained from fluorescence resonance energy transfer experiments (Colvert et al., 1992).

Colvert et al. (1992) observed a small (~5%) but consistent increase in the fluorescence quantum yield of coumarinyl-maleimide attached to Cys63 upon addition of adenine nucleotides to the isolated β polypeptide, suggesting that ATP binding at the distant nucleotide binding site induced a conformational change which was propagated along to the N-terminal portion of the β polypeptide. In this study we have further examined this conformational event and show that it is the direct result of ADP, rather than ATP, binding to the β polypeptide. The existence of the ADP-induced fluorescence response has allowed us to obtain a direct measurement of the dissociation constant for ADP binding to the β polypeptide.

The importance of the N-terminal domain of the β polypeptide in the catalytic process has also been further highlighted by recent studies (Avni et al., 1992) which have shown that an aspartic acid residue at position 83 on β may be at or close to the site of interaction between CF_1 and the potent allosteric inhibitor tentoxin. We (Hu et al., 1993) have further shown that tentoxin blocks cooperative interactions between at least two nucleotide binding sites on CF_1. Thus the N-terminal domain of β may play a critical role in conformational coupling of nucleotide binding sites during the catalytic process.

MATERIALS AND METHODS

Chemicals. TNP-ATP analogs and fluorescent maleimide derivatives were purchased from Molecular Probes Inc. Nonfluorescent nucleotides were from Sigma Chemical Co., and quinine sulfate was obtained from Aldrich Chemical Co. Other chemicals were of high-quality commercial grades, and all aqueous solutions were prepared with deionized, distilled water.

Preparation of CF_1 and the β Polypeptide. CF_1 was prepared from fresh market spinach (Binder et al.,1978; Lien & Racker, 1971; Hu et al., 1993) and stored as an ammonium sulfate precipitate. Prior to use CF_1 was desalted on a 1.5 × 15 cm column of Sephadex G-50 or using Sephadex G-50 centrifuge columns (Penefsky, 1977). The β polypeptide was prepared from CF_1 lacking the δ and ε polypeptides as described elsewhere (Richter et al., 1985). The protein was concentrated to >1 mg/mL in a dialysis membrane using Aquacide II (Calbiochem) and stored at −70 °C in the presence of 20% (v/v) glycerol and 1 mM ATP.

Chemical Modification of the β Polypeptide. The single β sulfhydryl was routinely modified with fluorescent maleimides by incubation of β with a 2-fold excess of the maleimide at room temperature for 15−30 min. The modification medium contained 25 mM tricine-NaOH (pH 8.0), 1 mM ATP, and 10−20 μM β polypeptide. Unreacted probes were removed by passing the labeled protein successively through two 3 mL Sephadex G-50 centrifuge columns. The extent of labeling was determined using the extinction coefficient of 3.02×10^4 M^{-1} cm^{-1} at 387 nm for CPM (Sippel, 1981) and 3.3×10^4 M^{-1} cm^{-1} at 343 nm for PM (Holowka & Hammes, 1977) after correction for light scattering. Protein concentrations were determined by the method of Bradford (1976).

Spectroscopic Measurements. Absorbance measurements were made with a Beckman DU-70 spectrophotometer. Steady state fluorescence measurements were made with a Perkin Elmer MPF-44B fluorescence spectrophotometer equipped with a DCSU-2 instrument for corrected spectrum. Quantum yields for the fluorescent-labeled β samples were calculated by the comparative method of Parker and Reese (1966) using quinine sulfate as standard. The quantum yield of quinine sulfate was assumed to be 0.70 (Scott et al., 1970).

Quenching of pyrenylmaleimide bound to Cys63 of the β polypeptide was performed by titrating acrylamide from a 6 M stock solution to 2 μM labeled β in 50 mM tricine-NaOH (pH 8.0) and observing the decrease in fluorescence emission at 374 nm with a 2 nm slit width (343 nm excitation, 2 nm slit width). After correcting for dilution effects, Stern−Volmer quenching constants (K_{Sv}) were obtained from plots of the initial fluorescence (F_o) divided by the fluorescence with quencher present versus the concentration of quencher where:

$$F_o/F = 1 + K_{Sv}[Q] \text{ and } K_{Sv} = k_q\tau \quad (1)$$

Bimolecular quenching constants (k_q) were calculated from the measured average weighted lifetime (τ) of the probe on the β polypeptide in the absence of quencher (see below).

The amount of TNP-nucleotide bound to the β polypeptide at different TNP-ATP or TNP-ADP concentrations was determined from the extent of fluorescence enhancement (λ_{ex} = 415 nm, λ_{em} = 550 nm) upon TNP-nucleotide binding as previously described (Mills & Richter, 1991). The quantum yield increase of fluorescent maleimides bound to Cys63 of the β polypeptide was measured upon addition of nucleotides. To correct for dilution and inner filter effects, the heat-denatured β polypeptide, which is no longer capable of binding nucleotides (Mills & Richter, 1991), was titrated with TNP-ATP/ADP and the resulting fluorescence was subtracted from that of the nondenatured protein.

Time-resolved fluorescence measurements were made using an ISS K2 frequency domain fluorometer equipped with a Marconi signal generator (2022A and C) and ENI broad-band amplifiers (325LA and 403LA) which operate in conjunction with a Pockels cell to sinusoidally modulate the light intensity. Excitation of β-PM used the 351 nm line from a continuous wave (CW) argon ion laser (Coherent Innova 400/2/0.3), and fluorescence emission was collected using an Oriel band-pass filter centered at 395 nm with a full width at half-maximum (Γ) of 8.8 nm. Phase and modulation data were measured relative to the reference glycogen (scatter, τ = 0 ns). The cell temperature was maintained at 16 °C with constant stirring. The sample lifetime was determined by the relative phase shift (ϕ_w) and demodulation (m_w) of the fluorescence at each modulation frequency as previously described (Spencer & Weber, 1969; Yao et al., 1994). The data were collected over a range of modulation frequencies and fit to multiexponential decay models using nonlinear least squares analysis (Beechem et al., 1991). Subsequent to the measurement of the intensity decay, one typically calculates the average lifetime, τ, which is weighted by the amplitudes associated with each of the

pre-exponential terms, where:

$$\tau = \sum_i \alpha_i \tau_i \quad (2)$$

τ is directly related to the average time the fluorophore is in the excited state, and the amplitude weighting implies a direct relationship between τ and the quantum yield of the fluorophore (Luedtke et al., 1981). Alternatively, the fluorescence intensity decay was sometimes fit using more realistic physical models involving a Lorentzian distribution of fluorescence lifetimes, as previously described (Alcala et al., 1987a).

Frequency domain measurements of the anisotropy decays of pyrenylmaleimide were determined by measuring the phase angle difference and the ratio of the modulated amplitudes between parallel and perpendicular components of the fluorescence emission. The measured values were fitted to an experimental decay model using the sine and cosine transformations of the individual polarized decays (Weber, 1981), where anisotropy decays are described as a sum of exponentials (Lakowicz, 1983) using the following multiexponential model:

$$r(t) = (r_0 - r_\infty)\sum g_i \exp(-t/\phi_i) + r_\infty \quad (3)$$

$r_0 g_i$ are the amplitudes associated with each component of the decay, ϕ_i are the rotational correlation times, r_0 is the initial anisotropy at time zero, and r_i is the residual anisotropy that does not decay during the excited lifetime of the fluorophore. The goodness of fit is estimated using the nonlinear least squares method (Johnson and Faunt, 1992):

$$\chi^2_R = 1/\nu[\sum_w(\Delta_w - \Delta_{cw}/\delta\Delta)^2 + \sum_w(\Lambda_w - \Lambda_{cw}/\delta\Lambda)^2] \quad (4)$$

where ν is the number of degrees of freedom, Δ_{cw} and Λ_{cw} are the calculated values for the phase difference and the ratio of the modulated amplitudes, respectively, and $\delta\Delta$ and $\delta\Lambda$ are the experimental uncertainties in the phase angle difference and the ratio of the modulation anisotropies.

Steady state fluorescence resonance energy transfer measurements were performed as described elsewhere (Snyder & Hammes, 1984, 1985). The efficiency of energy transfer was obtained from the relationship:

$$E = 1 - Q_{DA}/Q_D = 1 - \tau_{DA}/\tau_D \quad (5)$$

where Q_{DA}/Q_D and t_{DA}/t_D are the ratios of the donor quantum yields and fluorescent lifetimes, respectively, in the presence (DA) and absence (D) of acceptor compounds. The distances between the donor and acceptor sites were calculated from the relationship:

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

where N_A is the number of acceptors contributing to the quenching of a single donor, R_i is the distance between the donor and the ith acceptor, and R_o is the distance at which the transfer efficiency is 0.5 for a single donor−acceptor pair. Equation 2 assumes that each acceptor is present at a stoichiometry of 1 mol of acceptor/mol of β polypeptide. In eq 2, R_o is given by the expression:

$$R_o = (9.79 \times 10^3)(\kappa^2 J Q_D \eta^{-4})^{1/6} \quad (7)$$

where η is the refractive index of the medium, J is the spectral overlap integral, and κ^2 is an orientation factor for dipolar coupling between donor and acceptor (Förster, 1959). The value of κ^2 is assumed to be $^2/_3$, the dynamic average. The maximum uncertainties in the calculated distance due to this assumption are likely to be no worse than $\pm 10\%$ (Snyder & Hammes, 1985; Richter et al., 1985).

For titrations involving TNP-nucleotides and fluorescent maleimide derivatives located at Cys63, the contributions of inner filter effects and trivial transfer to the steady state quenching ratios were measured directly as described previously (Colvert et al., 1992). These corrections added less than 5% to the distance measurement and were similar to the inner filter correction used by Lakowicz (1983). TNP-ADP acceptor binding was extrapolated to a stoichiometry of 1 TNP-ADP bound/β polypeptide as before (Colvert et al., 1992).

RESULTS

Nucleotide-Induced Enhancement of Pyrenylmaleimide Fluorescence. Addition of ATP or ADP to the β polypeptide significantly enhanced the fluorescence of probes attached to Cys63. We first observed this effect as an approximately 5% enhancement of the fluorescence of coumarinylmaleimide (CPM) attached to this site (Colvert et al., 1992). However, the quantum yield of CPM-Cys63 approaches the maximum value of 1 under our experimental conditions, thus limiting the extent of enhancement obtained. To optimize the effect, different probes were substituted for CPM. Stilbenylmaleimide gave the largest enhancement (>25%), but the fluorescence decayed continually during the steady state measurements, presumably as a consequence of instability of the probe. A smaller (>10%) but stable response was obtained using pyrenylmaleimide (PM), and all subsequent measurements were made using this probe. It was also noticed that micromolar concentrations of ADP could elicit the fluorescence enhancement (Figure 1A, top data set), whereas previously it had been seen that millimolar concentrations of ATP were required (Colvert et al., 1992). Inclusion of an ATP-regenerating system with the added ATP totally abolished the ATP-induced effect (Figure 1A, lower data set) indicating that it was due to contaminating ADP. Thus the effect is due to ADP binding.

The fluorescence of PM attached to Cys63 was enhanced to a similar extent upon binding of either ADP or GDP but not CDP which was shown previously (Mills & Richter, 1991), not to bind to the isolated β polypeptide. Mild heat treatment, which is known to destroy nucleotide binding to the β polypeptide (Mills & Richter, 1991), abolished the ADP-induced fluorescence enhancement (Figure 1B). A Scatchard analysis of the concentration dependence for the ADP-induced fluorescence enhancement is shown in Figure 2A and compared to a similar analysis for GDP binding (Figure 2B). Dissociation constants of 1.05 μM for ADP and 16 μM for GDP were obtained from these data.

To determine whether or not ATP binds to the same site as ADP on the β polypeptide, we examined the ability of ATP to inhibit the ADP-induced enhancement of PM fluorescence. Indeed, ATP, at micromolar concentrations, blocked the ADP-induced fluorescence enhancement in a

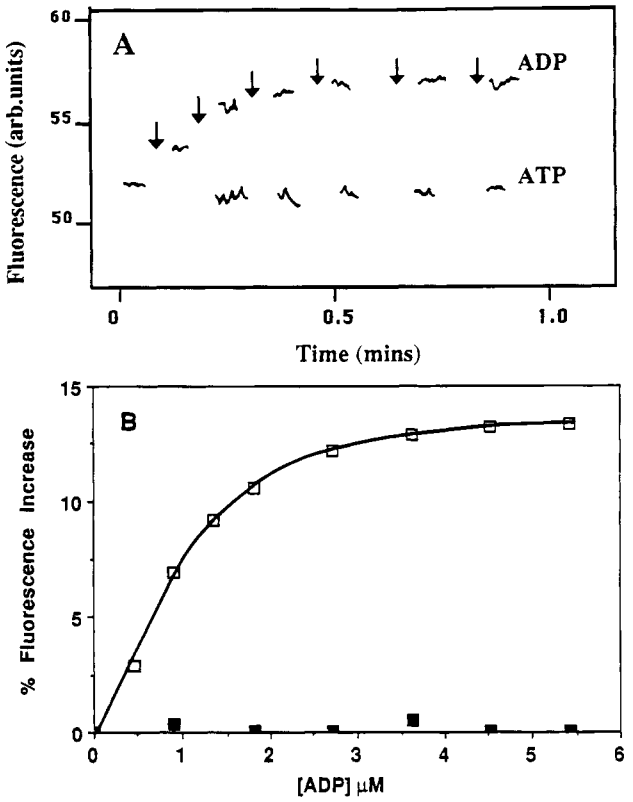

FIGURE 1: ADP-specific increase in β-PM fluorescence. The fluorescence of pyrenylmaleimide (PM) attached to Cys63 of the β polypeptide was monitored at 375 nm (excitation 343 nm) in 50 mM tricine-NaOH (pH 8.0), 20 units/mL pyruvate kinase, 5 mM phosphoenolpyruvate (PEP), and 1 μM β polypeptide at 15 °C. The PEP was ommitted from the incubation mixture when ADP was added. (A) Incremental additions of ADP (0.45 μM each, upper data set) or ATP (50 μM each, lower data set) were made at the times indicated by the arrows. (B) The fluorescence enhancement of β-PM upon addition of ADP is expressed in terms of percent fluorescence increase on β (□) and the β polypeptide which had been heat-denatured (■) by prior incubation at 40 °C for 15 min. This data have been corrected for inner filter and dilution effects.

competetive manner (Figure 3), indicating that both ADP and ATP bind to the same site but that only ADP induces the fluorescence change. From this data, an apparent K_d of 2 μM for ATP binding was calculated.

We previously reported K_d values for TNP-ADP and TNP-ATP binding to β in the range of 0.5−1 μM for both nucleotides (Mills & Richter, 1991; Chen et al., 1992). In those experiments, millimolar concentrations of ATP or ADP were required to displace significant amounts of the bound TNP-nucleotides, suggesting that ATP and ADP bound to the β polypeptide with much lower affinity than their TNP counterparts. The discrepancy between that observation and the micromolar K_d values obtained for ADP and ATP in this study could result from a large difference between the on-rate (faster) and the off-rate (slower) for TNP-nucleotide binding to the β polypeptide. A significant difference between these two rates was indicated previously (Grubmeyer & Penefsky, 1981; Hisabori et al., 1992). Thus we reinvestigated the ability of ADP or ATP to compete for TNP-nucleotide binding, but this time ADP or ATP was added to the β polypeptide prior to addition of TNP-ADP (Figure 4). Dissociation constants of 46 μM for ADP and 117 μM for ATP were calculated using a double-reciprocal analysis of the data shown in Figure 4. As expected these values are

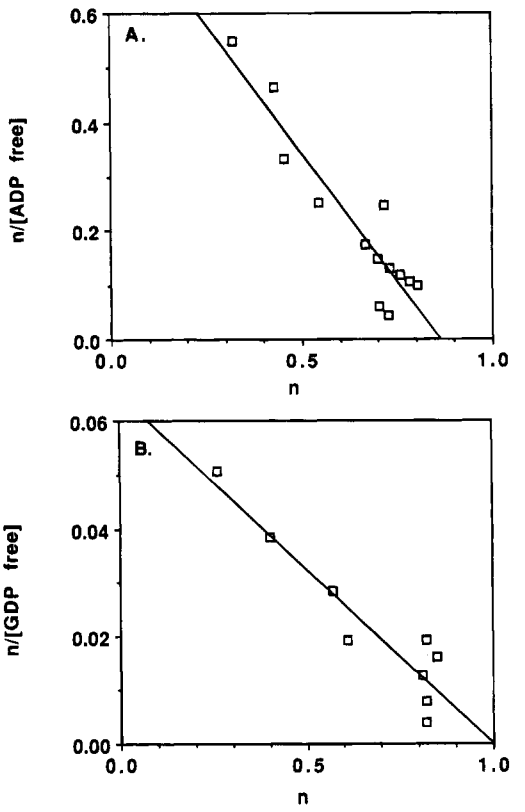

FIGURE 2: Scatchard analysis of nucleotide binding to β-PM. The effects of increasing concentrations of ADP (A) or GDP (B) on β-PM fluorescence were monitored under the conditions described in the legend to Figure 1 and in the methods section. The number of moles of nucleotide bound was calculated assuming a single nucleotide binding site on the β polypeptide (Mills & Richter, 1991). n is the number of mol of nucleotide bound/mol of β polypeptide.

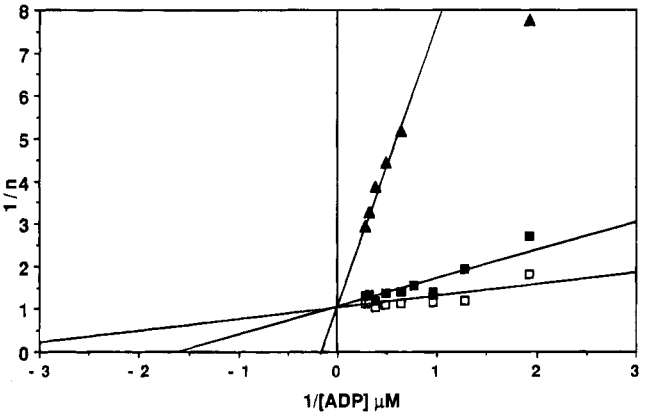

FIGURE 3: Competition between ATP and ADP for binding to the β polypeptide. β Cys63-PM fluorescence emission was monitored following addition of ADP as described in the legend to Figure 1: (□), no additions, (■), in the presence of 2 μM ATP, (Δ), in the presence of 10 μM ATP. n is the number of mol of nucleotide bound/mol of β polypeptide.

significantly lower than those indicated in our previous study, although they are still overestimates.

The fluorescence quantum yield for PM attached to β Cys63 was 0.56 as compared to values ranging between 0.18 and 0.26 previously observed for the probe attached to sulfhydryls of the γ polypeptide of CF_1 (Snyder & Hammes, 1985). This very high value for β-PM indicated that the probe was located in a very nonpolar environment. The increase in quantum yield observed upon ADP binding suggests a change in the local environment surrounding the

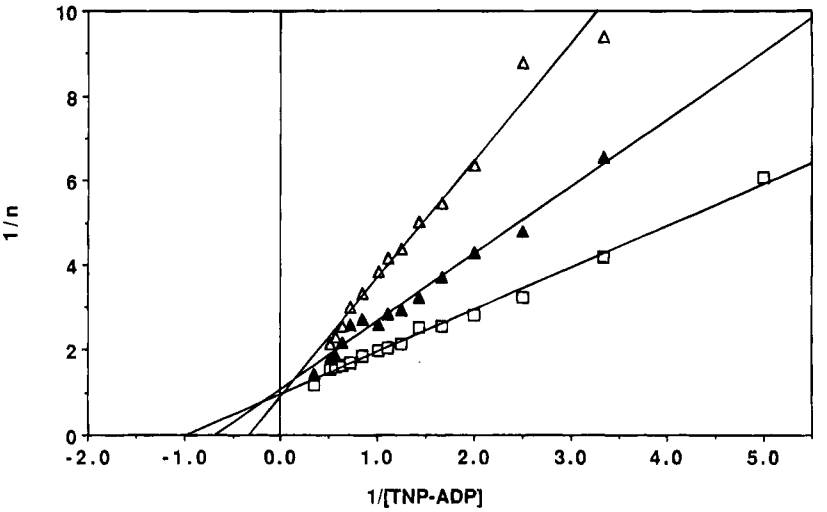

FIGURE 4: Competition between TNP-ADP, ATP, or ADP for binding to the β polypeptide. Enhancement of fluorescence emission of TNP-ADP upon binding to the β polypeptide (2 μM β in 1 mL of 50 mM tricine-NaOH, pH 8.0), was measured as described in the methods section. The data are shown as double-reciprocal plots of the TNP-ADP binding curves in the absence (□) or presence of 100 μM ATP (Δ) or 100 μM ADP (▲).

probe, most likely a further reduction in polarity since there was no significant shift in the emission spectrum of PM upon ADP binding.

Measurement of Fluorescence Lifetimes. We have measured the fluorescence decay of β-PM in order to investigate the structural alterations within the β polypeptide that are associated with nucleotide binding. Utilizing frequency domain fluorescence spectroscopy to measure the lifetime of β-PM, we measured the phase lag and demodulation of intensity-modulated light at 14 frequencies between 0.4 and 20 MHz. As the modulation frequency was increased we observed that the phase lag increased while the modulation decreased (Figure 5). In all cases the intensity decay of β-PM was adequately described as a sum of 3 exponentials which gave a greater than 2-fold improvement in the goodness of fit (i.e., χ^2_R) over a model involving two exponentials (not shown). This indicated that the three-exponential model was statistically justifiable. Inclusion of additional fitting parameters resulted in a minimal further improvement in χ^2_R. These results were used to calculate the average lifetime values listed in Table 1.

Changes in fluorescence lifetimes reveal structural changes in the nearby environment of fluorophores. There was a 9.5-fold increase in the average lifetime of PM upon covalent association with Cys63, indicating that β-PM is in a nonpolar environment. The average lifetime of β-PM increased by an additional 60% upon ADP binding, indicating that the probe had moved into an even more hydrophobic environment (Lakowicz, 1983). A similar increase in the average lifetime was observed upon mild heat treatment which was previously shown to selectively alter the conformation of the nucleotide binding site on the β polypeptide (Mills & Richter, 1991). This suggests that small structural changes associated with a conformationally unstable domain in the nucleotide binding site functions to trigger the structural change around Cys63 that decreases the polarity around the PM.

To further investigate the fluorescence lifetime changes, we fit the data (Figure 5) to a model assuming a Lorentzian distribution of fluorescence lifetimes, as previously described (Alcala et al., 1987a,b). This analysis explicitly attempts to

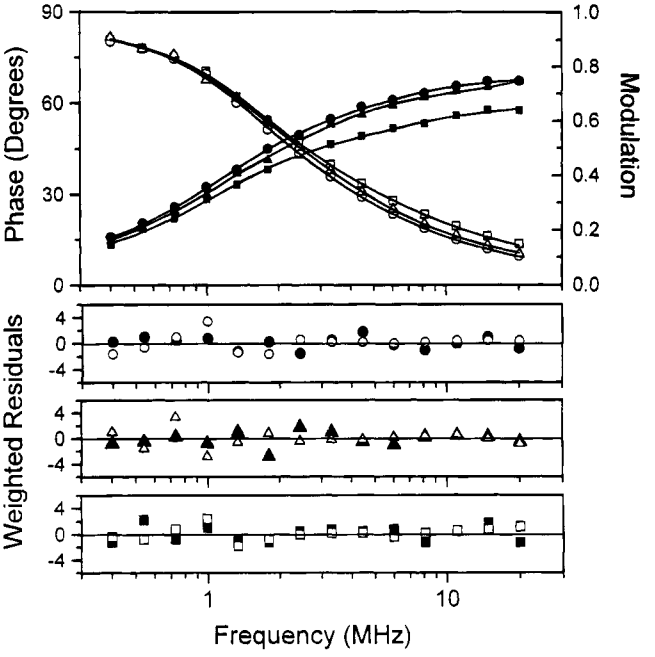

FIGURE 5: Frequency domain fluorescence response of β Cys63-PM. In all cases the lifetime decay of PM on the β polypeptide was measured at 14 frequencies between 0.4 and 20 MHz, and the phase lag (●, ▲, ■) and modulation (○, Δ, □) were adequately fit to a sum of three exponentials, as judged by the weighted residuals which are the calculated differences between the experimental data and the fit divided by the assumed error of the measurement. The assumed errors are 0.4° and 0.005 for the phase and the modulation, respectively (Gratton et al., 1984: Lakowicz et al., 1984). Measurements involved 2 μM β labeled with PM without additions (□, ■), in the presence of 1 mM ADP (○, ●), and subsequent to mild heat treatment (Δ, ▲). The buffer included 50 mM tricine-NaOH (pH 8.0). The associated χ^2_R for these samples are 8.2, 5.4, and 4.3, respectively. The temperature was 16 °C.

account for time-dependent alterations in the environment surrounding the fluorophore. Two lifetime components centered near 3 and 90 ns with associated half-widths (full width at half-height) of 8 and 48 ns are required to adequately describe the data (Figure 6). We found that there was a 2-fold reduction in the χ^2_R relative to that observed in the multiexponential model, suggesting that the model is statisti-

Table 1: Quenching[a] of β-PM Fluorescence by Acrylamide

	K_{SV} (M^{-1})[b]	τ (ns)[c]	χ^2_R[d]	k_q ($\times 10^{-9}$ M^{-1} s^{-1})[e]	$k_q/k_{q_{free}}$
PM free	2.34	3.9[f]		0.60	1.000
β-PM	0.60	37.4	1.5 (8.1)	0.016	0.027
β-PM + heat	0.50	54.3	2.0 (4.3)	0.009	0.015
β-PM + ADP	0.49	60.5	1.6 (5.4)	0.008	0.013
β-PM + ATP	0.62				

[a] Collisional quenching was used to measure the solvent accessibility of PM (see eq 1 in the methods section). [b] K_{SV} is the Stern−Volmer quenching constant at λ_{ex} = 343 nm and λ_{em} = 375 nm. [c] τ is the average fluorescence lifetime (see eq 2 in the methods section), using λ_{ex} = 351 nm. [d] χ^2_R describes the goodness of fit for the average lifetime data using a three-exponential model. The corresponding χ^2_R values for a two-exponential model are given in parentheses. [e] K_q is the bimolecular quenching constant, and $k_{q_{free}}$ is that obtained for PM attached to *N*-acetylcysteine, using acrylamide as the quenching agent. [f] Yao et al. (1994).

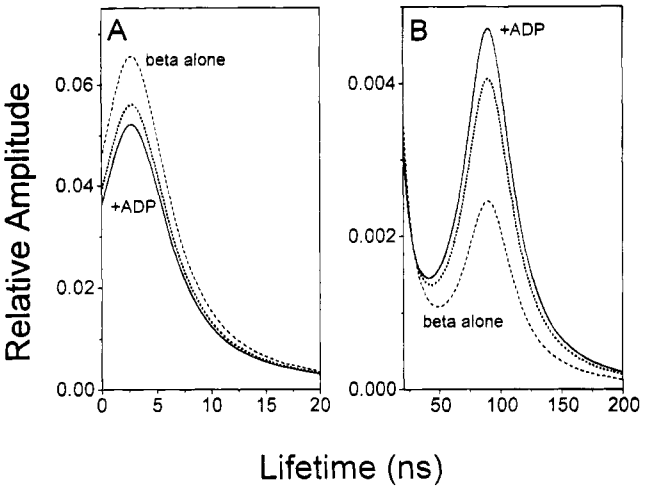

FIGURE 6: Ligand-dependent changes in conformational heterogeneity. The distribution of fluorescence lifetimes associated with both the short (A) and long (B) lifetime components of PM-labeled β polypeptide are illustrated for β alone (dashed line), β in the presence of 1 mM ADP (solid line), and mild-heat-treated β (dotted line). The associated χ^2_R values for these fits to a bimodal Lorentzian distribution of lifetimes are 3.4, 3.2, and 2.8, respectively.

cally valid. There was no corresponding improvement in χ^2_R when a model involving a Gaussian distribution of lifetimes was used to fit the experimental data. The associated lifetime center and the half-widths of these distributed components were similar for all three data sets, and the primary change associated with ligand binding involves alterations in the relative amplitudes of these lifetime components. Therefore, in order to more accurately determine these amplitudes, the associated centers and the half-widths of the distributions were linked during the fitting procedure (Beecham et al., 1991). It was observed that upon ADP binding the contribution of the long-lived component centered near 90 ns increases, consistent with the observed increase in quantum yield. Similarly, mild heat treatment was associated with an analogous increase in the long-lived component, suggesting that the disruption of the nucleotide binding domain results in an alteration in protein structure associated with the distant PM at Cys63. Given the large spatial separation (i.e., 42 Å; Colvert et al., 1992) between the nucleotide binding site and PM located at Cys63, which is normally located at the α−β interface on CF_1, these results provide evidence in favor of a long-range coupling mechanism.

Nucleotide-Induced Changes in Probe Accessibility. Fluorescence quenching studies supported the observation that ADP binding induced a change in the local environment surrounding β-PM. A Stern−Volmer constant of 0.60 M^{-1} was obtained for acrylamide quenching of PM bound at β Cys63 as compared to a value of 2.34 M^{-1} for PM (reacted with *N*-acetylcysteine) free in solution (Table 1), indicating that the bound probe was relatively inaccessible to solvent. In the presence of ADP, the Stern−Volmer constant decreased further to 0.50 M^{-1}, again indicating a further decrease in solvent accessibility. Using the average lifetime values (τ; Table 1), the bimolecular quenching constants (k_q) were calculated for the acrylamide quenching of PM. A 37-fold reduction in the efficiency of quenching was observed upon association of PM with β, consistent with the positioning of PM into a hydrophobic cleft within the polypeptide. The solvent accessibility of β-PM decreased 2-fold upon the addition of ADP or after heat denaturation of the β polypeptide, indicating that there was a substantial reduction in the solvent accessibility of PM upon heat denaturation or addition of ADP to the β polypeptide. It is expected that the k_q value of a probe will be reduced to a value close to 0.5 upon attachment of the probe to a protein. Any further reduction should reflect a decreased accessibilty of the probe to the solvent (Eftink, 1991). The low bimolecular quenching values obtained for PM attached to β Cys63 suggest that the probe had moved further into the protein interior where it was less accessible to solvent. Attempts to repeat these experiments with the more hydrophilic quenching agent potassium iodide were unsuccessful due to a large static component.

Rotational Dynamics of the β Polypeptide. In order to define possible structural changes associated with the global conformation of β, we used frequency domain measurements of fluorescence anisotropy of β-PM to measure the overall rotational motion which is sensitive to the overall dimensions of the protein. Data were collected over 23 frequencies from 0.4 to 10 MHz (not shown). Using algorithms previously described (Weber, 1981), we found that a sum of two exponentials was required to adequately describe the data as judged by both the 2−3-fold reduction in χ^2_R and the evenly distributed residuals (not shown). Inclusion of additional fitting parameters results in no significant improvement in the calculated fit to the data. No fast component due to motion of the probe on the protein was observed suggesting that the probe is in a restricted environment. Using the PM fluorophore, we were able to directly measure the hydrodynamic properties of β, permitting us to assess its overall dimensions [reviewed by Steiner (1991)]. For comparison, the expected rotational correlation time for an equivalent hydrated globular protein of molecular weight 54 kDa whose partial specific volume is 0.73 cm^3/g is 24 ns, where:

$$\varphi_1 = \eta V/kT$$

and η is the viscocity of water (1.111 cP at 16 °C), *V* is the hydrated volume of β (9.29×10^4 $Å^3$, assuming two shells of hydration; Cantor & Schimmel, 1980), *k* is the Boltzman's constant, and T is the absolute temperature (289 K). We found that the correlation time associated with the rotational mobility of β was 49 ± 8 ns and that upon ADP binding the correlation time did not change (Table 2). This indicates

Table 2: Rotational Correlation Time[a] of Isolated β Subunit

additions	$g_1 r_o$	ϕ_1 (ns)[a]	χ^2_R[b]
none	0.179	49 ± 8	1.86 (3.64)
1 mM ADP	0.204	47 ± 5	1.91 (2.88)
mild-heat-treated	0.135	88 ± 20	1.94 (4.22)

[a] Rotational correlation time (ϕ_1) associated with global rotational motion of isolated β polypeptides involved the measurement of the phase shift and the modulation of the fluorescence associated with pyrenyl maleimide covalently linked to Cys63 at 23 separate frequencies between 0.4 and 10 MHz. [b] χ^2_R describes the deviations between the model and experimental data for a two-component model involving a hindered rotator, as described in the methods section. The numbers in parentheses represent the χ^2_R for a one-component fit to the data. The initial anisotropy (r_o) for all three data sets was 0.218. The confidence interval associated with the correlation time was obtained from a rigorous error analysis of the errors associated with the measurement of the β rotational dynamics and accounts for all correlations between the measured parameters. In this analysis, the correlation time is systematically varied, and all other parameters are varied to optimize the fit to the data (i.e., the normalized χ^2 is minimized). The uncertainty associated with an increase in χ^2_N of one standard deviation (i.e., the *F*-statistic) defines the maximal possible error associated with the determination of the rotational correlation time. Excitation was the 351 nm line of an argon ion laser. Fluorescence emission was collected using an Oriel band-pass filter centered at 395 nm with a half-width of 8.8 nm at a protein concentration of 2 μM in 50 mM tricine-NaOH (pH 8.0) at 16 °C.

that (i) β is a nonspherical molecule and (ii) the overall dimensions of β do not significantly change upon ADP binding. Mild heat treatment substantially increased the rotational correlation time of β, suggesting that the protein underwent a conformational change which enhanced the frictional coefficient which is associated with rotational motion. If one assumes that β can be approximated by a prolate ellipsoid, this corresponds to a greater than 40% increase in the axial ratio.

Distance between β Cys63-PM and TNP-ADP. The fluorescence emission of PM overlaps significantly with the absorption spectrum of TNP-ADP (Figure 7) making them a potential donor−acceptor pair for fluorescence resonance energy transfer distance measurements. The calculated R_O for this pair is 39.5 Å (Table 3). From an observed energy transfer of 44% between the two probes, a distance of 42.7 Å was calculated. This value is in close agreement with the measurement of 41.8 Å obtained between coumarinyl-maleimide at Cys63 and TNP-ADP on the β polypeptide reported previously (Colvert et al., 1992).

DISCUSSION

The nucleotide-induced change in fluorescence yield of PM bound at Cys63 allowed, for the first time, an accurate calculation of the dissociation constants for ADP and ATP binding to the β polypeptide. ADP bound a little more tightly than ATP, but both nucleotides bound significantly more tightly than suggested earlier (Mills & Richter, 1991) from competition experiments with TNP-nucleotides bound at the same site. The observed dissociation constants of 1.05 and 2 μM for ADP and ATP, respectively, are very close to the Michaelis constant for ATP hydrolysis at the second of two (or possibly three) catalytic sites on CF_1 [reviewed by Boyer (1993)]. As pointed out previously (Mills & Richter, 1991), the nucleotide binding properties of the single site

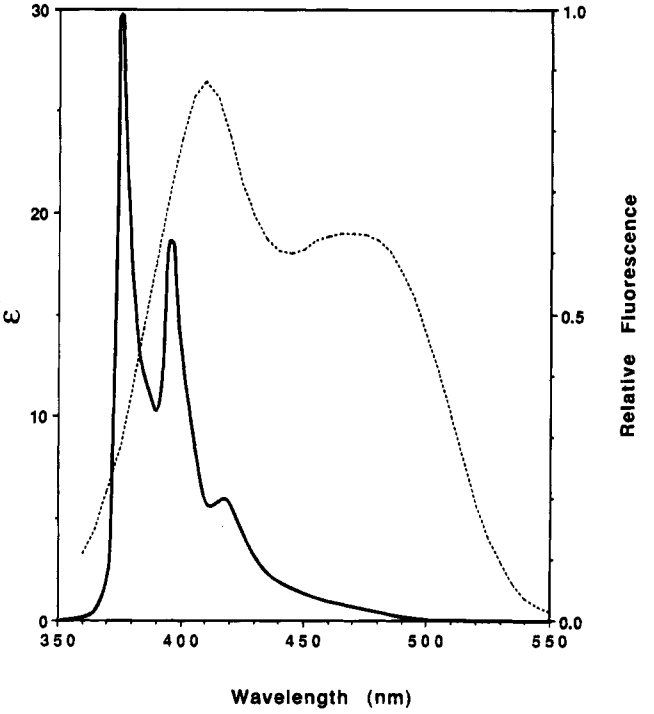

FIGURE 7: Spectral overlap of PM and TNP-ADP. The solid line is the fluorescence emission (excitation 343 nm) of PM covalently attached to the β polypeptide. The dashed line is the extinction coefficient (ϵ) for TNP-ADP bound at the nucleotide binding site of the β polypeptide. Both spectra were recorded in 50 mM tricine-NaOH (pH 8.0) at 23 °C.

on the isolated β polypeptide are nearly identical to those of the low-affinity site 3 on CF_1 characterized and mapped by Hammes and co-workers (Snyder & Hammes, 1984). Site 3 is considered to be a catalytic site.

Several other sites on CF_1 bind nucleotides with a much higher affinity than site 3 and are considered to be either noncatalytic sites or alternative "tight" conformations of catalytic sites. These sites are operationally defined as those which remain occupied by nucleotides following passage of the protein through one or more Sephadex centrifuge columns (Shapiro & McCarty, 1991). In our experience (D. Mills, unpublished experiments), such tight sites have dissociation constants of less than about 0.3 μM. Presumably, formation of tight nucleotide binding sites involves cooperative participation of the α and β polypeptides and probably also the γ polypeptide. It has been suggested that each catalytic site may cycle through two or three different conformational states during a single catalytic cycle (Boyer, 1993). One of these conformational states (the loose or open state) may involve minimal interaction between the β polypeptide and other polypeptides such that its nucleotide binding properties are essentially identical to those of the free β polypeptide.

The data presented in this paper have shown that there is a conformational coupling between the N-terminal region of the β polypeptide and the nucleotide binding site which is about 42 Å away. β Cys63 is already in a relatively nonpolar environment as indicated by the accessibility of quenching agents and the high quantum yields of fluorescent probes attached to this residue. The increase in quantum yield of pyrenylmaleimide, which occurred upon addition of ADP, suggested that Cys63 was moved into an even more hydrophobic environment. This movement was likely to involve a localized change in the structure of the β polypep-

Table 3: Fluorescence Resonance Energy Transfer Parameters[a]

donor	emission max (nm)	Q_D	acceptor	excitation max (nm)	J ($\times 10^{-14}$ M^{-1} cm^{-1})	R_0 (Å)	E
PM	375	0.56	TNP-ADP	415	4.46	39.48	0.438

[a] The R_0 (distance at 50% efficiency of energy transfer), energy transfer (E), the spectral overlap integral (J) calculated at 5 nm wavelength intervals for both emission of donor and absorption of acceptor, and the quantum yield of the donor (Q_D) were calculated as described in the methods section.

tide in the vicinity of Cys63, since nucleotide binding did not induce a significant change in the rotational correlation time of the probe, ruling out large global changes in the structure. Such a localized change may have resulted, for example, from movement of one or more hydrophobic amino acid residues into closer juxtaposition with the fluorescent probes attached to Cys63, thus decreasing the polarity of the local environment surrounding the probes. This would explain the relatively small decrease in fluorescence quenching by acrylamide which was observed upon nucleotide binding.

Anisotropy measurements of β-PM indicated that the isolated β polypeptide is nonspherical. Assuming that the shape of the protein can be approximated by an ellipse, the observed correlation time reflects an axial ratio close to 5. This value is consistent with an elongated, irregularly shaped polypeptide similar to the recently published crystal structure of β in the intact F_1 complex (Abrahams et al., 1994), indicating that the isolated β has an analogous structure to that observed within the enzyme complex.

Our data have shown that ADP (or TNP-ADP) and ATP bind to the same site on the β polypeptide with approximately equal affinities, yet only ADP induces the structural change in the protein which results in perturbation of the region around Cys63. The presence of the γ phosphate of ATP apparently blocks the conformational change. The ADP-induced conformational change is likely to be mechanistically important. ADP (and not ATP) induces an acceleration of the decay of the light-triggered state of ATPase in chloroplasts in the absence of phosphate (Carmeli & Lifshitz, 1972). This regulatory effect may occur by inducing conformational changes which increase the affinity for nucleotides at other sites (Dunham, 1981). The effect is prevented by addition of inorganic phosphate or reversed by imposition of a transmembrane proton gradient (Bickel-Sandkotter & Strotmann, 1981; Schumann, 1984; Fromme & Gräber, 1990). Tight binding of ADP also forces isolated CF_1 into an inactive conformation (Hammes, 1983).

The recent high-resolution structure of the bovine heart mitochondrial F_1 (Abrahams et al., 1994) has shown that the N-terminal portion of both the α and β polypeptides form β barrel domains separated from the main bulk of the proteins by random coil segments which potentially act as flexible hinges. The crystal structure has indicated that the six β barrels of the N-termini of α and β polypeptides form a nearly continuous β sheet structure which is largely responsible for holding the α and β polypeptides together (Abrahams et al., 1994). This region of the N-terminus is well-conserved among β polypeptides of F_1 enzymes from different sources, and so the N-terminal domain of the chloroplast β is likely to adopt a similar, if not identical, conformation. Thus β Cys63 would be located within the β barrel in β sheet "d". This β sheet in the bovine heart F_1 crystal structure is reported as being hydrogen-bonded to the "a" β sheet on the adjacent β barrel of the α subunit. The "d" sheet is located in the lower portion of the β barrel domain in a crevice which separates it from the larger nucleotide binding domain. This site is distant from the nucleotide binding site, in close agreement with our fluorescence energy transfer measurements.

In some recent experiments (Z. Chen, unpublished), we mutated the β N-terminal residue, Cys63, to tryptophan and found that the bulkier residue blocked ATP synthesis by a hybrid *Escherichia. coli* chloroplast F_1-F_0 complex. Miki et al. (1994) have observed similar effects upon substitution of *E. coli* β Leu40 and β Glu41 (equivalent to spinach CF_1 β Cys63 and Glu64, respectively) to bulkier amino acids. In fact, changing *E. coli* β Leu40 to proline blocked assembly of both α and β polypeptides, whereas changing *E. coli* β Glu41 to lysine blocked assembly of the α polypeptide into the F_1-F_0 complex. These results argue strongly for an involvement of the N-terminal domains of both α and β polypeptides in assembly, coupling, and catalysis. Moreover, Miki et al. showed that a reversion mutation at position 218 of the *E. coli* β polypeptide (equivalent to chloroplast β residue 246) restored function to the Lys41 mutant, indicating that these two residues are close to each other in the F_1 complex and that the enzyme is very sensitive to changes in this region of the β polypeptide. That Arg218 is in close proximity to Glu41 is supported by the crystal structure of the bovine heart enzyme which indicates that Arg218 (Arg 220 in bovine heart F_1) is located at the top of the main domain of the β polypeptide (Abrahams et al., 1994) where it could easily interact with Glu41 (Glu48 in bovine heart F_1).

On the basis of the observed ADP-induced fluorescence enhancement of probes attached to Cys63, we suggest that ADP binding induces a change in the structure such that the N-terminal domain might alter its contact with the main body of the β polypeptide. In the CF_1 complex, such a change could affect the interaction between the β polypeptide and the adjacent α polypeptides, thus propagating information to adjacent nucleotide binding sites. An altered interaction between the "a" and "d" β sheets could cause the N-terminal domains of the α and β polypeptides to rotate relative to each other. This may explain the different structure observed in crystals of rat liver mitochondrial F_1 which depicts the α subunits on a lower plane than the β polypeptides and tilted inward with respect to the β polypeptides (Bianchet et al., 1991).

A recent study by Avni et al. (1992) suggested that Asp83 on the β polypeptide is located at or close to the site at which the inhibitor tentoxin binds to CF_1. However, tight binding of tentoxin requires the presence of at least the α and β polypeptides (Avni et al., 1992; Hu et al., 1993). It was further shown that tentoxin blocked cooperative binding changes between two nucleotide binding sites on CF_1 (Hu et al., 1993). On the basis of these observations, we proposed that tentoxin binds at a site which is located between one particular α−β polypeptide pair on CF_1 and interferes with

an essential conformational interaction between the two polypeptides. Tentoxin may, therefore, act by binding in this region and blocking the $\alpha-\beta$ interaction. Binding of a single molecule of tentoxin to CF_1 is sufficient to completely block catalytic activity (Steele et al., 1976).

In summary, the results of this study have indicated that ADP binding to the β polypeptide imposes a conformational change in the N-terminal portion of the molecule across a distance of approximately 42 Å. Such conformational coupling between the two sites is likely to be of relevance to events taking place in the normal catalytic cycle of CF_1 in which nucleotide binding at one catalytic site induces a change in the nucleotide binding properties of one or more other catalytic sites through cooperative interactions among the different α and β polypeptides. These changes may be driven in reverse by the proton gradient during ATP synthesis by F_1-F_0 ATP synthase. Our current studies involve introduction of intrinsic tryptophan probes into the β polypeptide using site-directed mutagenesis of an overexpression clone of the *atpB* gene encoding the chloroplast β polypeptide, (Chen et al., 1992). With additional fluorescent reporter groups located at specific sites on the β polypeptide we hope to clarify the structural relationship between the nucleotide binding site and other regions of the molecule, especially the N-terminal domain which appears to play an essential role in the catalytic/coupling process.

REFERENCES

Abrahams, J. P., Leslie, A. G. W., Lutter, R., & Walker, J. E. (1994) *Nature 370*, 621−628.

Alcala, J. R., Gratton, E., & Prendergast, F. G. (1987a) *Biophys. J. 51*, 587−596.

Alcala, J. R., Gratton, E., & Prendergast, F. G. (1987b) *Biophys. J. 51*, 597−604.

Avni, A., Anderson, J. D., Holland, N., Rochaix, J.-D., Gromet-Elhanan, Z., & Edelman, M. (1992), *Science 257*, 1245−1247.

Beechem, J. M., Gratton, E., Ameloot, M., Knutson, J. R., & Brand, L. (1991) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) pp 241−305, Plenum Press, New York.

Bianchet, M., Xavier, Y., Hullihen, J., Pedersen, P. L., & Amzel, M. (1991) *J. Biol. Chem. 266*, 21197−21201.

Bickel-Sandkotter, S., & Strotmann, H. (1981) *FEBS Lett. 125*, 188−192.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094−3100.

Boekema, E. J., & Bottcher, B. (1992) *Biochim. Biophys. Acta 1098*, 131−143.

Boyer, P. D. (1993) *Biochim. Biophys. Acta 1140*, 215−250.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298−6305.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. 2, W.H. Freeman and Co., San Francisco, CA.

Carmeli, C., & Lifshitz, Y. (1972) *Biochim. Biophys. Acta 267*, 86−95.

Cerione, R. A., & Hammes, G. G. (1982) *Biochemistry 21*, 745−752.

Chen, Z., Wu, I., & Richter, M. L. (1992) *FEBS Lett. 298*, 69−73.

Colvert, K., Mills, D. A., & Richter, M. L. (1992) *Biochemistry 31*, 3930−3935.

Duncan, T. M., Parsonage, D., & Senior, A. E. (1986) *FEBS Lett. 208*, 1−6.

Dunham, K. R. (1981) in *Energy Coupling in Photosynthesis* (Selman, Selman-Reimer, Eds.) pp 209−221, Elsevier North Holland, The Netherlands.

Eftink, M. R. (1991) in *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy* (Dewey, T. G., Ed.) pp 1−41, Plenum Press, NY.

Förster, T. (1959) *Discuss. Faraday Soc. 27*, 7−17.

Fromme, P., & Gräber, P. (1990) *Biochim. Biophys. Acta 1016*, 29−42.

Garboczi, D. N., Shenbagamurthi, W. K., Hullihen, J., & Pedersen, P. L. (1988) *J. Biol. Chem. 263*, 812−816.

Gratton, E., Lakowicz, J. R., Maliwal, B., Cherek, H., Laczko, G., & Linkeman, M. (1984) *Biophys J.* 46, 479−486.

Grubmeyer, C., & Penefsky, H. S. (1981) *J. Biol. Chem. 256*, 3718−3727.

Harris, D., & Nadanaciva, S. (1989) *Physiol. Plant. 76*, A162.

Hisabori, T., Muneyuki, E., Odaka, M., Yokoyama, K., Mochizuki, K., & Yoshida, M. (1992) *J. Biol. Chem. 267*, 4551−4556.

Hammes, G. G. (1983) *TIBS 8*, 131−134.

Holowka, D. A., & Hammes, G. G. (1977) *Biochemistry 16*, 5538−5545.

Hu, N., Mills, D. A., Huchzermeyer, B., & Richter, M. L. (1993) *J. Biol. Chem.* 268, 8536−8540.

Johnson, M. L., & Faunt, L. M. (1992) *Methods Enzymol. 210*, 1−37.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

Lakowicz, J. R., Gratton, E., Laczko, G., Cherek, H., & Linkeman, M. (1984) *Biophys. J. 46*, 463−477.

Leckband, D., & Hammes, G. G. (1987) *Biochemistry 26*, 2306−2312.

Lien, S., & Racker, E. (1971) *Methods Enzymol. 23a*, 547−555.

Luedtke, R., Owen, C. S., Vanderkooi, J. M., & Karush, F. (1981) *Biochemistry 20*, 2927-2936.

McCarty, R. E., & Moroney, J. V. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) 2nd ed., pp 383−413, Plenum Press, New York.

Miki, J., Tsugumi, S., & Kanazawa, H. (1994) *Arch. Biochem. Biophys. 312*, 317−325.

Mills, D. A., & Richter, M. L. (1990) in *Current Progress in Photosynthesis Research* (Baltcheffsky, M., Ed.) Vol. III, pp 49−52, Kluwer Academic Publishers, Dordrecht.

Mills, D. A., & Richter, M. L. (1991) *J. Biol. Chem. 266*, 7440−7444.

Nalin, C. M., & Nelson, N. (1987) *Curr. Top. Bioenerg. 15*, 273−294.

Ohta, S., Tsuboi, M., Oshima, T., Yoshida, M., & Kagawa, Y. (1980) *J. Biochem. 87*, 1609−1617.

Parker, C. A., & Reese, W. T. (1966) *Analyst (London) 85*, 587−600.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891−2899.

Richter, M. L., Snyder, B., McCarty, R. E., & Hammes, G. G. (1985) *Biochemistry 24*, 5755−5763.

Richter, M. L., Gromet-Elhanan, Z., & McCarty, R. E. (1986) *J. Biol. Chem. 261*, 12109−12113.

Schumann, J. (1984) *Biochim. Biophys. Acta 766*, 334−342.

Scott, T. G., Spencer, R. D., Leonard, M. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687−695.

Shapiro, A. B., & McCarty, R. E. (1988) *J. Biol. Chem. 263*, 14160−14165.

Shapiro, A. B., & McCarty, R. E. (1990) *J. Biol. Chem. 265*, 4340−4347.

Shapiro, A. B., & McCarty, R. E. (1991) *J. Biol. Chem. 266*, 4194−4200.

Shapiro, A., Gibson, K. D., Scheraga, H., & McCarty, R. E. (1991) *J. Biol. Chem. 266*, 17276−17285.

Sippel, T. O. (1981) *J. Histochem. Cytochem. 29*, 1377−1381.

Snyder, B., & Hammes, G. G. (1984) *Biochemistry 23*, 5787−5795.

Snyder, B., & Hammes, G. G. (1985) *Biochemistry 24*, 2324−2331.

Spencer, R., & Weber, G. (1969) *Ann. N. Y. Acad. Sci. 158*, 361−376.

Steele, J. A., Uchytil, T. F., Durbin, R. D., Bhatnagar, P., & Rich, D. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2245−2248.

Steiner, R. F. (1991) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) Vol. 2, pp 1−52, Plenum Press, New York.

Weber, G. (1981) *J. Phys. Chem. 85*, 949−953.

Yao, Y., Schoneich, C., & Squier, T. (1994) *Biochemistry 33*, 7797−7810.

Xue, Z., Zhou, J.-M., Melese, T., Cross, R. L., & Boyer, P. D. (1987) *Biochemistry 26*, 3749−3753.

BI942510+