Small, D. M. (1967) *J. Lipid Res. 8*, 551-557.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711-733.
Torbet, J., & Wilkins, M. H. F. (1976) *J. Mol. Biol. 62*, 447-458.
van Olphen, H. (1977) *An Introduction to Clay Colloid Chemistry*, 2nd ed., p 216, Wiley-Interscience, New York.
Vink, H. (1971) *Eur. Polym. J. 7*, 1411-1419.
White, S. H., & King, G. I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6532-6536.
Worcester, D. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359-378.
Worthington, C. R., King, G. I., & McIntosh, T. J. (1973) *Biophys. J. 13*, 480-494.

Chloroplast Coupling Factor 1: Dependence of Rotational Correlation Time on Polypeptide Composition†

Jeffrey E. Schinkel and Gordon G. Hammes*
Department of Chemistry, Cornell University, Ithaca, New York 14853-1301
Received January 13, 1986; Revised Manuscript Received February 4, 1986

ABSTRACT: Time-resolved fluorescence depolarization measurements were made on chloroplast coupling factor 1 (CF_1) labeled with pyrenylmaleimide. Rotational correlation times were determined for native CF_1, for CF_1 lacking ϵ and/or δ polypeptides, and for activated enzyme. The rotational correlation time measured is characteristic of the rotation of the entire enzyme. Removal of the δ polypeptide resulted in a 25% smaller rotational correlation time, although the δ polypeptide contributes less than 5% of the mass of CF_1. Removal of the ϵ polypeptide was without effect. Simultaneous removal of δ and ϵ polypeptides produced a 30% smaller rotational correlation time. Activation of CF_1 ATPase by incubation with dithiothreitol reduced the rotational correlation time by 15% relative to that of the latent enzyme. The rotational correlation time of CF_1 with δ and ϵ polypeptides removed is essentially that expected for a spherical molecule, whereas the other forms of the enzyme can be approximated as ellipsoids of revolution; the axial ratio of the latent enzyme is estimated from the rotational correlation time and the intrinsic viscosity. These data indicate that the δ polypeptide significantly alters the shape of the enzyme and that a conformational change accompanies dithiothreitol activation of the enzyme.

Chloroplast coupling factor is an integral membrane protein that catalyzes ATP synthesis by use of a transmembrane proton gradient. The extrinsic portion of the enzyme, CF_1,[1] is water-soluble and can be removed by treatment with EDTA (Lien & Racker, 1971). CF_1 is a latent ATPase comprised of five types of polypeptides with a total M_r of 400 000 (Moroney et al., 1983). The polypeptide stoichiometry is probably $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983).

Nucleotide binding and catalysis by CF_1 involve the α and β polypeptides (Kambouris & Hammes, 1985). The γ polypeptide is involved in regulation of catalysis, and it may be involved in proton translocation (McCarty & Moroney, 1985). Four cysteine residues have been identified on the γ polypeptide (Nalin & McCarty, 1984; Moroney et al., 1984). Two of the four cysteines form a disulfide linkage; reduction of the disulfide converts the latent ATPase to an active state. A change of conformation is associated with activation (Nalin & McCarty, 1984; Schumann et al., 1985). Of the two remaining cysteines, one is accessible under all conditions (dark site), while the other is reactive only under energized conditions with CF_1 bound to the thylakoid membrane (light site). The δ polypeptide is essential to the functional integrity of membrane-bound coupling factor (tight coupling), although it is not required for binding of CF_1 to the membrane (Patrie & McCarty, 1984). The ϵ polypeptide is a potent ATPase inhibitor and is closely associated with the γ polypeptide (Richter et al., 1985). The ϵ polypeptide contains one cysteine residue that can be modified by sulfhydryl reagents.

Detailed information about the structural organization of CF_1 has been obtained by fluorescence resonance energy transfer methods (Cerione & Hammes, 1982; Snyder & Hammes, 1984, 1985; Richter et al., 1985). These data have shown a structural asymmetry that reflects the configuration of the ϵ and γ polypeptides. This paper describes time-resolved fluorescence depolarization measurements that indicate an important contribution of the δ polypeptide to the shape of CF_1. Measurements made with activated enzyme show that reduction of the γ disulfide changes the conformation of the enzyme.

Materials and Methods

Chemicals. ATP (vanadium free) and dithiothreitol were from Sigma Chemical Co. Pyrenylmaleimide was from Molecular Probes, Inc. Hydroxylapatite (fast flow), octyl glucoside, and nonyl glucoside were from Calbiochem-Behring. DE-23 cellulose (advanced fibrous) was from Whatman. All other chemicals were high-quality commercial grades, and all solutions were prepared with deionized water.

CF_1 Preparation. CF_1 was prepared from fresh market spinach as previously described (Lien & Racker, 1971; Binder

† This work was supported by a grant from the National Institutes of Health (GM13292). J.E.S. is a National Institutes of Health Postdoctoral Fellow (GM09354).

[1] Abbreviations: CF_1, chloroplast coupling factor; CF_1-ϵ, chloroplast coupling factor lacking the ϵ polypeptide; CF_1-δ, chloroplast coupling factor lacking the δ polypeptide; CF_1-(δ,ϵ), chloroplast coupling factor lacking the δ and ϵ polypeptides; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-4066$01.50/0

et al., 1978). Purified enzyme was precipitated with an equal volume of saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.2) and 1 mM EDTA and stored at 4 °C. Concentrations of CF_1 were measured at 277 nm with an extinction coefficient of 0.483 mL/(mg·cm) (Bruist & Hammes, 1981) and a M_r of 400 000 (Moroney et al., 1983). Prior to measurements with native CF_1, the enzyme was subjected to the same ion exchange chromatography as CF_1-ϵ and CF_1-(δ,ϵ), except the elution step for δ and ϵ polypeptides was omitted.

CF_1-ϵ and CF_1-(δ,ϵ) were prepared by ion exchange chromatography (Richter et al., 1984) on an 0.5 (i.d.) × 7.0 cm column (Pasteur pipet). The eluants were as described except for inclusion of 100 mM NaCl during the elution of δ and ϵ polypeptides (Mark Richter, personal communication). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Chua, 1980) confirmed complete removal of δ and ϵ polypeptides for CF_1-(δ,ϵ), but some ϵ polypeptide (ca. 10%) always remained in preparations of CF_1-ϵ.

CF_1-δ was prepared by hydroxylapatite chromatography (Patrie & McCarty, 1984) on an 0.5 (i.d.) × 4.0 cm column (Pasteur pipet). The eluant included either 30 mM octyl glucoside or 10 mM nonyl glucoside. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated complete removal of δ polypeptide. The use of octyl glucoside, however, also resulted in partial removal of ϵ polypeptide, as recently reported (Yu & McCarty, 1985).

Activation of latent, native CF_1 was accomplished by incubation with 50 mM dithiothreitol for at least 3 h (Nalin & McCarty, 1984). Latent CF_1 deficient in ϵ polypeptide was activated by incubation with 20 mM dithiothreitol for a minimum of 30 min (Richter et al., 1985).

Chemical Modification of CF_1. Labeling of CF_1 always preceded chromatographic preparation of the different forms of the enzyme described above. The exposed γ polypeptide sulfhydryl (dark site) was specifically modified by reacting CF_1 (5–10 μM) with 1 μM pyrenylmaleimide for 30 s in 2 mM EDTA, 50 mM NaCl, and 10 mM Tris-HCl (pH 8.0). The reaction was stopped by passing the protein solution through two consecutive 3-mL centrifuge columns containing Sephadex G-50 equilibrated with the same buffer (Penefsky, 1977). The stoichiometry was always less than 0.20 pyrenylmaleimide per CF_1. In the case of CF_1-(δ,ϵ), a higher stoichiometry was possible because reaction with the ϵ polypeptide sulfhydryl was inconsequential. The conditions were as above, but 50 μM pyrenylmaleimide was allowed to react for 15 min. Stoichiometries ranged from 1.68 to 1.81 pyrenylmaleimides per CF_1 prior to removal of the ϵ polypeptide and from 0.84 to 0.90 after removal of the ϵ polypeptide.

Specificity of labeling was checked by visually examining the fluorescence of sodium dodecyl sulfate–polyacrylamide slab gels illuminated with near–ultraviolet light (Chua, 1980). The only observable fluorescence was associated with the γ polypeptide. (If the stoichiometry of pyrenylmaleimide to CF_1 exceeded 0.30 and ϵ polypeptide was still present, as was the case for some preliminary experiments, a faint, second fluorescent band was discernable at the position of the ϵ polypeptide.)

Pyrenylmaleimide labeling stoichiometry was calculated with an extinction coefficient of 3.75×10^4 M^{-1} cm^{-1} at 343 nm (Holowka & Hammes, 1977). Correction was made for CF_1 light scattering at 343 nm and for probe absorbance at 277 nm.

Concentrations of pyrenylmaleimide–labeled, polypeptide-deficient CF_1 [CF_1-δ, CF_1-ϵ, and CF_1-(δ,ϵ)] were determined by measurement of relative fluorescence (343-nm excitation, 375-nm emission). Sample fluorescence intensities were compared to the initial fluorescence intensity of labeled CF_1, whose concentration and pyrenylmaleimide labeling stoichiometry were known.

Ca^{2+}-ATPase Assay. Hydrolysis of [γ-^{32}P]ATP proceeded for 5 min at 37 °C in 5 mM $CaCl_2$, 5 mM ATP, 50 mM Tris-HCl (pH 8.0), and approximately 2 μg of CF_1. The assay volume was 0.5 mL, and the specific activity of radioactive ATP was 2 μCi/mL. The reaction was quenched by addition of 0.5 mL of 5% trichloroacetic acid, and then, 2.0 mL of 4% activated charcoal (suspended in water) was added. The solutions were thoroughly mixed by vortexing and then cleared of charcoal by passage through individual, disposable Millex-HA, 0.45-μm filters (Millipore Corp.). The amount of free ^{32}P was determined by measuring the Cerenkov radiation; 1.0 mL of sample was added to 10.0 mL of water, and the radioactivity was determined in a Beckman LS200 scintillation counter. Assays were performed in duplicate.

Control samples measured total specific activity (water added in place of charcoal) and background specific activity (protein added after trichloroacetic acid). Background counts were relatively high, up to 5% of the total activity, but this varied with labeled stocks, and nonspecific hydrolysis was negligible.

Spectroscopic Measurements. Absorbances were measured with a Cary 118C spectrophotometer. Steady-state fluorescence spectra and polarization measurements were made with an Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with polarization accessories. Correction was made for unequal transmission of vertically and horizontally polarized light by the emission monochromator grating (Cerione & Hammes, 1982).

Time-Resolved Fluorescence Measurements. Dynamic fluorescence measurements of pyrenylmaleimide-labeled CF_1 were made with an Ortec 9200 nanosecond fluorescence spectrophotometer previously described (Matsumoto & Hammes, 1975). Excitation light, provided by a spark-gap flash lamp, was passed through a Ditric Optics 340-nm (three-cavity) band-pass filter and was then vertically polarized. Fluorescence emission was either vertically or horizontally polarized, followed by passage through a Corning CS 0-51 filter, before detection by the photomultiplier. Background and scattering corrections were made by measuring the polarized emission from unlabeled CF_1 having the same concentration as labeled enzyme. Lamp-pulse profiles were obtained from the light scattering of a 0.1% Ludox solution (Du Pont), with the emission cut-off filter removed.

Instrumental fluctuations were corrected for in two ways (Waskiewicz & Hammes, 1982). The first approach was to regularly and frequently switch between collection of vertically and horizontally polarized emission fluorescence [$V(t)$ and $H(t)$]. The second method involved measuring intermediate ratios of $V(t)/H(t)$ at each switching. Then, before data analysis, the observed decays were normalized to the average of the ratios.

The time-resolved fluorescence $F(t)$ and anisotropy $r(t)$ were calculated as

$$F(t) = V(t) - V_s(t) + 2[H(t) - H_s(t)] \quad (1)$$

$$r(t) = [V(t) - V_s(t) - H(t) + H_s(t)]/F(t) \quad (2)$$

where the subscript s indicates spectra collected with unlabeled enzyme. The data were fit to the equations

$$F(t) = F_1 e^{-t/\tau_1} + F_2 e^{-t/\tau_2} \quad (3)$$

$$r(t) = r_0 e^{-t/\phi} \quad (4)$$

Table I: Fluorescence Properties of Pyrenylmaleimide-Labeled CF_1[a]

form of enzyme	F_1	τ_1 (ns)	F_2	τ_2 (ns)	r_0	ϕ (ns)
CF_1 (latent)	0.75	25.5	0.25	87.1	0.17	330 (3)
CF_1-ϵ (latent)	0.76	23.0	0.24	87.6	0.18	332 (2)
CF_1-δ (latent)	0.74	23.0	0.26	82.1	0.22	250 (2)
CF_1-δ (latent)[b]	0.78	19.7	0.22	84.1	0.20	240 (1)
CF_1-(δ,ϵ) (latent)	0.64	25.6	0.36	97.3	0.20	231 (4)
CF_1 (active)	0.72	23.4	0.28	81.7	0.17	280 (3)
CF_1-(δ,ϵ) (active)	0.67	27.7	0.33	105.4	0.17	262 (2)

[a] Experimental conditions are described under Materials and Methods. The fluorescence parameters are defined in eq 3 and 4, with $F_1 + F_2 = 1.00$. The rotational correlation times are the average of the number of experiments in parentheses with a standard deviation of less than 5%. [b] Plus 0.4 M NaCl.

where the τ_i's are fluorescence lifetimes, ϕ is the rotational correlation time, and F_1, F_2, and r_0 are amplitude parameters. A weighted, nonlinear least-squares analysis was used to analyze the data, with a numerical procedure to convolute the lamp-pulse profile (Munro et al., 1979; Siegel & Cathou, 1981). Normalized residuals were calculated as $(y_i - x_i)/x_i$, where y_i and x_i are the observed and calculated values, respectively, of $F(t)$ or $r(t)$. The goodness of fit was assessed by the reduced χ^2, where $\chi^2 = \sum_i w_i(y_i - x_i)^2/(n_d - n_p - 1)$. Here, n_p is the number of parameters, n_d is the number of data points, and w_i is a weighting factor (Bevington, 1969).

Protein concentrations were typically 1–2 μM. For CF_1 and CF_1-ϵ, the buffer was 2 mM ATP, 0.4 M NaCl, and 25 mM Tris-HCl (pH 7.9). For CF_1-(δ,ϵ), the buffer was 1 mM ATP, 1 mM EDTA, 225 mM NaCl, and 20 mM Tris-HCl (pH 7.9). For CF_1-δ, the buffer was 0.2 mM ATP, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.0). For measurements at increased viscosities, CF_1 solutions were diluted one to one with buffers containing the appropriate concentrations of glycerol. Correlation between percent glycerol and viscosity was based on data taken from the *CRC Handbook of Chemistry and Physics* (1973). Measurements were made at room temperature, 22 °C.

Results

Fluorescence Measurements. Pyrenylmaleimide was chosen as the fluorescent probe for two reasons. First, latent CF_1 has only two accessible sulfhydryl sites, one on the γ polypeptide and one on the ϵ polypeptide. Careful control of the reaction conditions allowed specific labeling of the γ polypeptide with the maleimide adduct; this label is near the center of the enzyme (Snyder & Hammes, 1985). Second, the pyrene moiety has a long average fluorescence lifetime that permits anisotropy measurements related to rotation of the overall molecule. Preliminary experiments indicated a need to use samples that had low label to enzyme stoichiometry, which avoided labeling of the ϵ polypeptide sulfhydryl, and to work with samples that were freshly prepared. The former ensured that the fluorescence properties were characteristic of a single environment. The latter condition was necessary because of aminolysis, a relatively slow, secondary reaction of pyrenylmaleimide characterized by deterioration of the fluorescence emission spectrum (Wu et al., 1976).

Typical fluorescence and anisotropy decay curves are shown in Figures 1 and 2. The normalized residuals also are shown. Analysis of the data with more than one rotational correlation time did not improve the fit of the data significantly. The fluorescence lifetimes and rotational correlation times obtained from CF_1, CF_1-ϵ, CF_1-δ, CF_1-(δ,ϵ), activated CF_1, and activated CF_1-(δ,ϵ) labeled with pyrenylmaleimide are summarized in Table I. The estimated uncertainty in the rotational correlation times is about ±5%. The values of the reduced χ^2 ranged from 4 to 9 for the analysis of the fluorescence and from 0.6 to 1.8 for the analysis of the anisotropy. The values

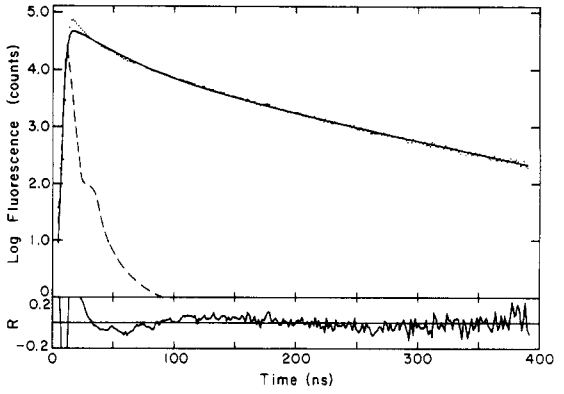

FIGURE 1: Plot of logarithm of total fluorescence of dithiothreitol-activated, pyrenylmaleimide-labeled CF_1 vs. time in 2 mM ATP, 0.4 M NaCl, and 25 mM Tris-HCl (pH 7.9) at 22 °C. The solid line was calculated with the best fit parameters obtained from eq 3. The dashed line is the lamp-pulse profile. The normalized residuals, R, are shown on the bottom, with a linear ordinate scale.

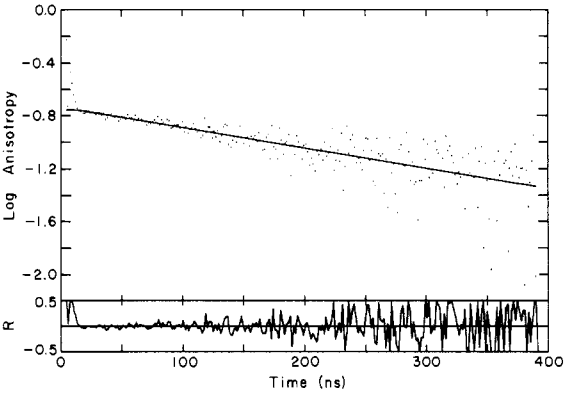

FIGURE 2: Plot of logarithm of anisotropy of dithiothreitol-activated, pyrenylmaleimide-labeled CF_1 vs. time in 2 mM ATP, 0.4 M NaCl, and 25 mM Tris-HCl (pH 7.9) at 22 °C. The solid line was calculated with the best fit parameters obtained from eq 4. The normalized residuals, R, are shown on the bottom, with a linear ordinate scale.

of χ^2 are smaller for the anisotropy analysis because the lamp deconvolution partially cancels when the anisotropy is calculated.

Fluorescence Anisotropy of Latent Enzyme. Removal of δ polypeptide resulted in a 25% decrease of the rotational correlation time of CF_1-δ compared with that of native CF_1 (Table I). A possible explanation for this result is the lower ionic strength that was a consequence of the procedure used to prepare CF_1-δ. This was shown not to be the case by measurement of the rotational correlation time of CF_1-δ under two sets of conditions. Measurement first was made with a sample in the usual manner, followed by measurement with the same sample brought into the ionic strength range of the CF_1, CF_1-ϵ, and CF_1-(δ,ϵ) samples by addition of NaCl (0.4 M final concentration). The consequence of the higher ionic strength was a slight (insignificant) decrease of the rotational

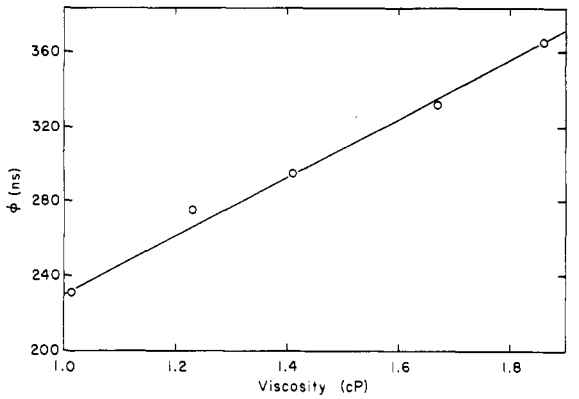

FIGURE 3: Plot of rotational correlation time of pyrenylmaleimide-labeled CF_1-(δ,ϵ) vs. viscosity in 1 mM ATP, 1 mM EDTA, 225 mM NaCl, and 20 mM Tris-HCl (pH 7.9) at 22 °C. The solid line was calculated with linear least-squares regression.

Table II: Dependence of Fluorescence Properties of CF_1-(δ,ϵ) on Viscosity[a]

viscosity (cP)	F_1	τ_1 (ns)	F_2	τ_2 (ns)	r_0	ϕ (ns)
1.86 (1.84)	0.63	34.5	0.37	106.4	0.19	365
1.67 (1.65)	0.68	21.5	0.32	96.0	0.18	332
1.41 (1.39)	0.65	22.0	0.35	93.5	0.19	295
1.23 (1.21)	0.64	20.4	0.36	93.0	0.19	275

[a] Samples of pyrenylmaleimide-labeled CF_1-(δ,ϵ) at different percent glycerol were prepared as described under Materials and Methods. The viscosity relative to that of the solvent in the absence of glycerol (1.013 cP) is in parentheses. The fluorescence parameters are defined by eq 3 and 4, with F_1 and F_2 = 1.00.

correlation time (Table I). Another explanation is the loss of ϵ polypeptide, resulting in CF_1-(δ,ϵ). However, analysis of samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated no loss of ϵ polypeptide.

Simultaneous removal of δ and ϵ polypeptides resulted in a 30% decrease of the rotational correlation time of CF_1-(δ,ϵ) compared with that of CF_1 (Table I). Furthermore, the rotational correlation time of CF_1-(δ,ϵ) was a linear function of the viscosity (Figure 3). This suggests the rotational correlation time is characteristic of the overall rotation of the molecule. For rotational diffusion of a sphere

$$\phi = \eta V/(kT) \quad (5)$$

where ϕ is the rotational correlation time, η is the viscosity, V is the hydrated molecular volume, k is the Boltzmann constant, and T is the temperature. Extrapolation of the data to zero glycerol concentration gave a rotational correlation time of 232 ns, in good agreement with independent measurements of the rotational correlation time for CF_1-(δ,ϵ) in the absence of glycerol (Table I). The apparent molecular volume obtained from the slope is 6.39×10^5 Å^3. The fluorescence lifetimes and rotational correlation times obtained at different viscosities are summarized in Table II.

The rotational correlation time of latent CF_1 was not affected by removal of ϵ polypeptide (Table I). Although a trace amount of ϵ polypeptide was present, measurements made at low pyrenylmaleimide to CF_1 stoichiometry effectively eliminated interference from secondary labeling of the ϵ polypeptide sulfhydryl. In contrast, preliminary experiments with samples labeled at high stoichiometry indicated an apparent decrease in rotational correlation time to 257 ns (data not shown). However, analysis of the samples by sodium dodecyl sulfate–polyacrylamide slab gels indicated labeling of both γ and ϵ polypeptides. When subsequent measurements were made with identically prepared samples having a low label to protein stoichiometry, the rotational correlation time of CF_1-ϵ remained unchanged compared with CF_1.

The ϵ polypeptide may affect rotational diffusion of CF_1 deficient in δ polypeptide. This was suggested by comparison of the rotational correlation times for CF_1-δ and CF_1-(δ,ϵ). However, the difference observed is close to the experimental uncertainty in the rotational correlation times.

Fluorescence Anisotropy of Activated Enzyme. Dithiothreitol activation of CF_1 resulted in a 15% decrease of the rotational correlation time (Table I). The specific activity of the active enzyme was about 11 μmol of P_i/(mg·min), both before and after the time-resolved fluorescence measurements.

Activation of CF_1-(δ,ϵ) with dithiothreitol resulted in a 12% increase relative to latent CF_1-(δ,ϵ) and a 7% reduction of the rotational correlation time relative to active CF_1 (Table I). Again, the ATPase activity, about 17 μmol of P_i/(mg·min), did not change during the course of the experiments.

Discussion

Two conclusions are apparent from inspection of the data in Table I. The first is that loss of the δ polypeptide causes a major change in the structure of CF_1. The second is that activation of enzyme results in a significant change of enzyme conformation.

Removal of δ polypeptide strongly affects the rotational correlation time of CF_1-δ compared with that of CF_1. Although some uncertainty remains, only a single copy of δ polypeptide is believed to be present in CF_1 (Moroney et al., 1983; McCarty & Moroney, 1985). The molecular weight of δ polypeptide is 17 500 (Nelson et al., 1973), which is less than 5% of the CF_1 mass. The simplest interpretation of the data is that CF_1 is nonspherical and that removal of δ polypeptide results in a more spherical structure. The structure of purified δ polypeptide has been reported to be highly asymmetric (Smith & Sternweiss, 1977; Schmidt & Paradies, 1977).

The smaller rotational correlation time for CF_1-δ is probably not simply due to a change in conformation of CF_1-δ relative to CF_1. CF_1 lacking δ polypeptide binds tightly to NaBr-treated thylakoids, although it cannot recouple ATP synthesis (McCarty & Moroney, 1985), and is fully active as an ATPase. The lack of recoupling activity has been attributed to proton "leakage" through CF_1-δ; native CF_1 is fully competent when bound. If purified δ polypeptide is added back to CF_1-δ, the reconstituted CF_1 restores photophosphorylation (Andreo et al., 1982). These results suggest that the conformation of CF_1-δ is essentially the same as CF_1 except for the absence of δ polypeptide.

Activation of CF_1 by equilibration with dithiothreitol has a significant effect on the rotational correlation time. Three possible explanations are denaturation of the enzyme resulting in segmental motion, dissociation of δ polypeptide from CF_1, and conformational changes associated with the activation. The first possibility is unlikely because the specific activity of the Ca^{2+}-ATPase was the same before and after the dynamic fluorescence measurements, about 11 μmol of P_i/(mg·min). The second possibility was tested by use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After the fluorescence measurements were completed, the samples were divided into two equal volumes. One portion was passed through a Sephadex G-50 centrifuge column (Penefsky, 1977), while the other portion served as a control. This treatment was sufficient to remove dissociated δ (or ϵ) polypeptide, but no difference in the intensity of the protein bands in the stained gel was observed between the two samples. Therefore, the smaller rotational correlation time is due to a conformational change associated with activation of the enzyme.

Activation of CF_1-(δ,ϵ) by equilibration with dithiothreitol resulted in a rotational correlation time slightly smaller than that of activated CF_1 and slightly greater than that of latent CF_1-(δ,ϵ); however, the differences are close to the uncertainties in the rotational correlation times. Like activated CF_1, the specific activity of CF_1-(δ,ϵ), 17 μmol of P_i/(mg·min), remained unchanged during the experiment.

Activation by equilibration with dithiothreitol was chosen because it is a reversible process (Arana & Vallejos, 1982; Nalin & McCarty, 1984), while proteolysis, heat activation, and other methods result in permanent changes in the enzyme (McCarty & Moroney, 1985). Reduction of the γ polypeptide disulfide results in a polypeptide conformation that is similar to that of activated, membrane-bound enzyme (Schumann et al., 1985). The rate of disulfide reduction (activation) depends on the presence of ϵ polypeptide, which has been located adjacent to the γ polypeptide (Richter et al., 1985), and activation may be mediated by interaction between ϵ and γ polypeptides. Therefore, the change to the active-state conformations of the ϵ and γ polypeptides could account for the smaller rotational correlation times. Although major conformational changes of the α and β polypeptides could be responsible for the smaller rotational correlation time, this seems unlikely since the rotational correlation time of activated CF_1-(δ,ϵ) is slightly larger than that of latent CF_1-(δ,ϵ).

The rotational correlation time for a hydrodynamic sphere can be calculated from eq 5 if the hydrated volume is known. The hydrated volume V can be estimated from the relationship

$$V = M_r(v + h)/N \quad (6)$$

where M_r is the molecular weight, v is the anhydrous specific volume, h is the hydration, and N is Avogadro's number. With M_r = 400000 (Moroney et al., 1983), v = 0.745 cm^3/g, and h = 0.59 or 0.65 cm^3/g (Paradies et al., 1978), the calculated rotational correlation time is 220–230 ns, which is significantly less than the value for the latent enzyme, 330 ns, and the activated enzyme, 280 ns. Apparently both species are somewhat asymmetric. The calculated value of the rotational correlation time for CF_1-(δ,ϵ) is 210–220 ns, which is quite close to the experimental value of 231 ns. Therefore, this species must be close to spherical. (The value of V obtained from the slope of the line in Figure 3 is somewhat smaller than that calculated from eq 6, $\sim 8.2 \times 10^5$ Å^3, but the value from the slope cannot be taken literally because of the unknown effect of glycerol.)

The axial ratio of an ellipsoid of revolution can be estimated from the deviation of the rotational correlation time from the value expected for a sphere. The best method for doing this is to combine the rotational correlation time with another hydrodynamic parameter such as the intrinsic viscosity (Scheraga, 1961). We have attempted to measure the intrinsic viscosity of CF_1 and CF_1-(δ,ϵ). However, the large amount of enzyme required and the sensitivity of enzyme to surface denaturation, especially CF_1-(δ,ϵ), prevented definitive results from being obtained. If, however, the intrinsic viscosity for latent CF_1 is estimated from the viscosity at 5.10 mg/mL as 4.18 mL/g, an axial ratio can be obtained (Scheraga, 1961). For a prolate ellipsoid, a/b = 1.7, and for an oblate ellipsoid, b/a = 5. Both prolate (Satre & Zaccai, 1979) and oblate (Paradies & Schmidt, 1979; Suss et al., 1978; Akey et al., 1983) ellipsoids have been used to describe the shape of coupling factors, usually with an axial ratio of about 2. At the present time, an unequivocal distinction between the two types of ellipsoids cannot be made. If the δ polypeptide is viewed as part of the stalk connecting CF_1 to the membrane, a prolate ellipsoid appears to be the better model. [This simplified analysis neglects the fact that ellipsoids are characterized by a multiexponential anisotropy decay [cf. Hammes (1981)]. Since the data are adequately fit by a single rotational correlation time, the assumption has been made that an effective rotational diffusion constant D can be calculated from the relationship $\phi = 1/(6D)$.]

Rotational correlation times for CF_1 have been determined recently with absorption anisotropy (Wagner et al., 1985). The rotational correlation times reported are consistently larger than those found in this work. Although a definitive explanation for these differences is not possible, we note that larger rotational correlation times are observed if the enzyme is not extremely pure or is somewhat denatured. In particular, small amounts of ribulose bisphosphate carboxylase can increase the rotational correlation time, and CF_1-(δ,ϵ) denatures very easily.

In summary, time-resolved fluorescence measurements have shown that latent CF_1 is nonspherical in shape but that removal of δ polypeptide results in an almost spherical molecule. Activation of CF_1 also causes a change in shape. The precise location of the δ polypeptide within CF_1 is currently being explored with fluorescence resonance energy transfer measurements.

Registry No. ATPase, 9000-83-3; dithiothreitol, 3483-12-3.

REFERENCES

Akey, C. W., Spitsberg, V., & Edelstein, S. J. (1983) *J. Biol. Chem. 258*, 3222–3229.

Andreo, C. S., Patrie, W. J., & McCarty, R. E. (1982) *J. Biol. Chem. 257*, 9968–9975.

Arana, J. L., & Vallejos, R. H. (1982) *J. Biol. Chem. 257*, 1125–1127.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

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

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

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

Chua, N.-H. (1980) *Methods Enzymol. 69*, 434–446.

CRC Handbook of Chemistry and Physics (1973) (Weast, R. C., Ed.) 54th ed., pp D205–206, CRC Press, The Chemical Rubber Co., Cleveland, OH.

Hammes, G. G. (1981) in *Protein-Protein Interactions* (Frieden, C., & Nichol, L. W., Eds.) pp 257–287, Wiley, New York.

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

Kambouris, N. K., & Hammes, G. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1950–1953.

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

Matsumoto, S., & Hammes, G. G. (1975) *Biochemistry 14*, 214–224.

McCarty, R. E., & Moroney, J. V. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 4, pp 383–413, Plenum, New York.

Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58–62.

Moroney, J. V., Fullmer, C. S., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7281–7285.

Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56–60.

Nalin, C. M., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7275–7280.

Nelson, N., Deters, D. W., Nelson, H., & Racker, E. (1973) *J. Biol. Chem. 253*, 2049–2055.

Paradies, H. H., & Schmidt, U. D. (1979) *J. Biol. Chem. 254*, 5257–5263.
Paradies, H. H., Zimmerman, J., & Schmidt, U. D. (1978) *J. Biol. Chem. 253*, 8972–8979.
Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 11121–11128.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Richter, M. L., Patrie, W. J., & McCarty, R. E. (1984) *J. Biol. Chem. 259*, 7371–7373.
Richter, M. L., Snyder, B., McCarty, R. E., & Hammes, G. G. (1985) *Biochemistry 24*, 5755–5763.
Satre, M., & Zaccai, G. (1979) *FEBS Lett. 102*, 244–248.
Scheraga, H. A. (1961) *Protein Structure*, Academic, New York.
Schmidt, U. D., & Paradies, H. (1977) *Biochem. Biophys. Res. Commun. 78*, 1043–1052.
Schumann, J., Richter, M. L., & McCarty, R. E. (1985) *J. Biol. Chem. 260*, 11817–11823.
Siegel, R. C., & Cathou, R. E. (1981) *Biochemistry 20*, 192–198.
Smith, J. B., & Sternweiss, P. C. (1977) *Biochemistry 16*, 306–311.
Snyder, B., & Hammes, G. G. (1984) *Biochemistry 23*, 5787–5795.
Snyder, B., & Hammes, G. G. (1985) *Biochemistry 24*, 2324–2331.
Suss, K.-H., Damaschun, H., Damaschun, G., & Zirwer, D. (1978) *FEBS Lett. 87*, 265–268.
Wagner, R., Engelbrecht, S., & Andreo, C. S. (1985) *Eur. J. Biochem. 147*, 163–170.
Waskiewicz, D. E., & Hammes, G. G. (1982) *Biochemistry 21*, 6489–6496.
Wu, C.-W., Yarbrough, L. R., & Wu, F. Y.-H. (1976) *Biochemistry 15*, 2863–2868.
Yu, F., & McCarty, R. E. (1985) *Arch. Biochem. Biophys. 238*, 61–68.

Interactions of *Neurospora crassa* Plasma Membrane H^+-ATPase with *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline†

Randolph Addison‡ and Gene A. Scarborough*
Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Received January 8, 1986; Revised Manuscript Received March 12, 1986

ABSTRACT: The carboxyl group activating reagent *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) interacts with the *Neurospora* plasma membrane H^+-ATPase in at least three different ways. This reagent irreversibly inhibits ATP hydrolysis with kinetics that are pseudo-first-order at several concentrations of EEDQ, and an appropriate transform of these data suggests that 1 mol of EEDQ inactivates 1 mol of the H^+-ATPase. Inhibition probably involves activation of an ATPase carboxyl group followed by a nucleophilic attack by a vicinal nucleophilic functional group in the ATPase polypeptide chain, resulting in an intramolecular cross-link. The enzyme is protected against EEDQ inhibition by MgATP in the presence of vanadate, a combination of ligands that has previously been shown to "lock" the H^+-ATPase in a conformation that presumably resembles the transition states of the enzyme phosphorylation and dephosphorylation reactions, but is not protected by the substrate analogue MgADP, which is consistent with the notion that one or both of the residues involved in the EEDQ-dependent inhibitory intramolecular cross-linking reaction normally participate in the transfer of the γ-phosphoryl group of ATP, or are near those that do. The ATPase is also labeled by the exogenous nucleophile [^{14}C]glycine ethyl ester in an EEDQ-dependent reaction, and the labeling is diminished in the presence of MgATP plus vanadate. However, peptide maps of [^{14}C]glycine ethyl ester labeled ATPase demonstrate that the labeling is not related to the EEDQ inhibition reaction in any simple way. In a third type of interaction, EEDQ mediates the specific cross-linking of ATPase monomers with some other membrane protein, possibly another ATPase monomer, leading to the formation of a product with an apparent molecular weight of about 260 000. This reaction occurs substantially more slowly than the inhibition reaction and is thus presumably not directly related either.

The principal ATP hydrolyzing enzyme in the plasma membrane of the filamentous fungus *Neurospora crassa* is an electrogenic proton pump (Scarborough, 1976, 1980) capable of generating a transmembrane electrical potential difference in excess of 200 mV (Slayman et al., 1973). The hydrolytic moiety of this enzyme has a molecular mass of about 105 000 daltons (Dame & Scarborough, 1980, Addison & Scarborough, 1981, Bowman et al., 1981), and recently, evidence has been provided indicating the strong likelihood that no subunits other than the hydrolytic moiety are involved in the catalysis of transport by this enzyme (Scarborough & Addison, 1984) and that monomers are efficient proton pumps (Goormaghtigh et al., 1986). The primary goal of this laboratory is an understanding of how this single polypeptide chain catalyzes electrogenic proton translocation at the expense of ATP hydrolysis. As part of our efforts in this regard, we have been investigating a variety of ATPase inhibitors as potential reagents for labeling the active site and other sites necessary for catalytic activity. With the knowledge that the β-carboxyl

† Supported by U.S. Public Health Service National Institutes of Health Grant GM24784.
‡ Present address: Laboratory of Cell Biology, The Rockefeller University, New York, NY 10021-6399.

0006-2960/86/0425-4071$01.50/0