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Rotational Dynamics of Chloroplast ATP Synthase in Phospholipid Vesicles†

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ABSTRACT: The rotational dynamics of the purified dicyclohexylcarbodiimide-sensitive H^+-ATPase (DSA) reconstituted into phospholipid vesicles and of the DSA coreconstituted with the proton pump bacteriorhodopsin were examined by using the technique of time-resolved phosphorescence emission anisotropy. The phosphorescent probe erythrosin isothiocyanate was used to covalently label the γ-polypeptide of DSA before reconstitution. Rotational correlation times were measured under a variety of conditions. The rotational correlation time was independent of the viscosity of the external medium but increased significantly as the microviscosity of the membrane increased. This indicates the rotational correlation times are a measure of the enzyme motion within the membrane. The activation energy associated with the rotational correlation time is 8-10 kcal/mol. At 4 °C, the correlation time, typically ~100-180 μs, was unaffected by the addition of substrates and the presence of a membrane pH gradient. Therefore, molecular rotation of the DSA does not appear to play an important role in enzyme catalysis or ion pumping.

The dicyclohexylcarbodiimide-sensitive H^+-ATPase (DSA)[1] from spinach chloroplasts is composed of a soluble portion, coupling factor 1 (CF_1), and a membrane component, CF_0. The entire protein complex consists of at least nine different polypeptide chains and is responsible for coupling the synthesis of ATP to the flow of protons across the thylakoid membrane. DSA can be isolated, purified, and reconstituted into phospholipid vesicles. The reconstituted enzyme catalyzes ATP-P_i exchange (Winget et al., 1977; Pick & Racker, 1979). While the exact mechanism for catalysis by the coupling factor is still not known, activation and catalysis clearly involve conformational changes in the CF_1 portion of the enzyme (Farron & Racker, 1970; Girault & Galmiche, 1977; Wagner & Junge, 1980; Bruist & Hammes, 1982; Pick & Finel, 1983; Leckband & Hammes, 1987; Shapiro & McCarty, 1988). Most of the evidence for these changes has been obtained by studying CF_1 in solution, and the dynamic motions of the more complex membrane-reconstituted DSA have not been thoroughly investigated.

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[1] Abbreviations: DSA, dicyclohexylcarbodiimide-sensitive H^+-ATPase; CF_1, coupling factor 1 of DSA; CF_0, coupling factor 0 of DSA; bR, bacteriorhodopsin; DPH, 1,6-diphenyl-1,3,5-hexatriene; ErITC, erythrosin isothiocyanate; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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The motions of biological molecules in fluid media typically occur in the nanosecond time domain and can, therefore, be studied with the techniques of fluorescence spectroscopy. In viscous media or biological membranes, however, motions are more restricted and typically take place in the micro- to millisecond time range. Therefore, the study of the rotational motions of membrane proteins often can be accomplished through measurements of time-resolved phosphorescence anisotropy [cf. Austin et al. (1979), Moore et al. (1979), and Greinert et al. (1979)].

In the present study, the dynamic properties of the purified DSA reconstituted into phospholipid vesicles were studied by the technique of time-resolved phosphorescence emission anisotropy. The phosphorescent probe ErITC was covalently and specifically attached to the CF_1 portion of the enzyme complex prior to reconstitution. The decay of anisotropy in the time range of 10–1000 μs was measured. The dependence of the rotational correlation time on the presence of substrates (ATP or ADP and P_i) and the effects of varying external medium viscosity, membrane viscosity, lipid:protein ratio, and temperature were determined. The dynamic motion of the DSA coreconstituted with the proton pump bR also was investigated. Anisotropy measurements were performed in the presence and absence of light with the bR–DSA vesicles. The light activates bR and creates a proton gradient across the membrane. The rotational correlation time could, therefore, be measured under conditions of ATP synthesis.

Materials and Methods

Materials. ErITC and DPH were purchased from Molecular Probes. Octyl β-D-glucopyranoside was obtained from Calbiochem. Cholic acid, recrystallized prior to use (Kagawa & Racker, 1971), ATP (vanadium free), ADP, Triton X-100, dithiothreitol, cholesterol, glucose oxidase, catalase, and asolectin (crude soybean phospholipids) were purchased from Sigma Chemical Co. [^{32}P]P_i was obtained from ICN. All other chemicals were high-quality commercial grades, and all solutions were prepared from deionized water.

Preparation and Labeling of DSA. Partially purified DSA was prepared from fresh market spinach as previously described (Pick & Racker, 1979; Cerione et al., 1983). ErITC labeling was achieved as follows. Immediately before use, a stock solution of ErITC was prepared to 1 mg/mL in 0.4 M sucrose, 10 mM NaCl, and 50 mM Tricine, pH 8.3. Thylakoids in the same buffer (40 mL) were prepared from 250 g of spinach and were incubated for 20 min with 60 μM ErITC added in small aliquots during the incubation period. To quench the reaction, glycine was added from a 1 M stock solution to a final concentration of 10 mM. The remainder of the preparation was performed under dimmed lights to avoid photobleaching of the probe. Further purification of both labeled and unlabeled DSA was accomplished by sucrose density centrifugation (Cerione et al., 1983). Fractions of 0.2 mL were collected at 4 °C from the top of the gradient and stored at −70 °C. Only the most concentrated fractions (13–16) which were free of the major contaminent, ribulose-1,5-bisphosphate carboxylase, were used for anisotropy measurements.

Protein concentrations were determined by a modification of the Lowry method (Bensadoun & Weinstein, 1976) with bovine serum albumin as a standard. Reconstituted DSA (see below) was assayed for ATP–P_i exchange activity according to Winget et al. (1977), except that bovine serum albumin was omitted from the reaction medium. Specific exchange activities for purified labeled and unlabeled DSA preparations were 150–240 nmol of P_i/(mg·min) (37 °C).

Labeling stoichiometries were determined by assuming an extinction coefficient of 83 000 M^{-1} cm^{-1} at 530 nm for ErITC (Moore et al., 1979; Restall et al., 1984). Specificity of labeling was checked by examining the fluorescence of ErITC–DSA bands on sodium dodecyl sulfate–polyacrylamide gels with ultraviolet light before staining. Stoichiometries were kept low (<1 mol of ErITC/mol of DSA) to minimize nonspecific labeling.

Preparation of Reconstituted DSA. DSA reconstitution into phospholipid vesicles was performed essentially as described by Krupinski and Hammes (1986). Asolectin liposomes were prepared to a concentration of 5–15 mg/mL in 150 mM KCl, 2 mM EDTA, and 10 mM Na-Tricine, pH 8.0 (KTE buffer). The suspension was mixed with a vortex under nitrogen and sonicated to clarity in a batch-type sonicator. In some preparations, 19–37 mol % cholesterol was incorporated into the phospholipid vesicles as follows. A maximum of 0.5 mg of solid cholesterol and solid asolectin (molar quantities based on an average molecular mass of 578 g/mol) were combined in the desired molar ratio and dissolved in 0.5 mL of chloroform. This mixture was evaporated to dryness under nitrogen and redissolved in KTE buffer to a final concentration of 8 mg/mL. The suspension was mixed with a vortex under nitrogen and sonicated to clarity as described above. The following ice-cold solutions then were added to purified DSA on ice: 6.0 μL of 0.5 M $MgCl_2$ (8.6 mM final concentration), 6–18 μL of 20% sodium cholate (3.5–10.5 mg/mL final concentration), 175 μL of sonicated asolectin or cholesterol/asolectin (2.5–7.5 mg/mL final concentration), and KTE buffer to a final volume of 350 μL. This mixture was eluted through a 3-mL centrifuge column packed with Sephadex G-50 fine preequilibrated in KTE buffer containing 3 mM $MgCl_2$ and 0.3% glucose. In most experiments, 35% sucrose also was added to the column buffer, and one experiment was done in the presence of 50% glycerol. Dithiothreitol then was added to the reconstituted DSA to a final concentration of 50 mM, and the enzyme was kept on ice until use. In some experiments, ATP (3 mM) or ADP (3 mM) and P_i (3 mM) were added from concentrated stock solutions to the reconstituted DSA sample.

For the preparation of bR–DSA vesicles, bR was first reconstituted into phospholipid vesicles as described by Krupinski and Hammes (1986). This preparation then was used for the DSA reconstitution performed as described above. Dithiothreitol was not added to these coreconstituted vesicles; however, hexokinase (10 units/mL) and glucose (30 mM) were added from concentrated stock solutions. bR from *Halobacterium halobium* strain S9-P, a gift from Dr. Aaron Lewis, Cornell University, was isolated and purified by Bharati Mitra as previously described (Krupinski & Hammes, 1985).

Steady-State Fluorescence Anisotropy Measurements. Steady-state fluorescence measurements were performed at 4 °C and 25 °C using a Perkin-Elmer MPF-44B fluorescence spectrophotometer. Asolectin suspensions containing either 0, 26, 31, or 37 mol % cholesterol were prepared as described above. Labeling of lipids with DPH was accomplished by addition of 6 μL of DPH from a 1 mM stock solution in acetonitrile to 500 μL of the cholesterol/lipid suspension. This corresponds to an approximate DPH to lipid ratio of 1:1000. Each preparation then was deoxygenated under nitrogen and sonicated in a bath-type sonicator for 10–20 min. For fluorescence measurements, samples were diluted to 0.4 mg/mL. Anisotropy measurements were not started until DPH labeling was complete (about 2 h after addition), as judged by a constant value of the DPH fluorescence emission

at 430 nm (excitation 350 nm). For each sample, the intensity of the DPH fluorescence emission at 430 nm (excitation 350 nm) was determined with vertically polarized excitation light and both vertically and horizontally polarized emission. An instrumental correction factor (G factor) also was determined by measuring vertically and horizontally polarized fluorescence emission intensity with horizontally polarized excitation. The steady-state anisotropy, r, then was calculated by using the equation:

$$r = \frac{GI_{VV} - I_{VH}}{GI_{VV} + 2I_{VH}}$$

where I_{VV} and I_{VH} are the emission components polarized parallel and perpendicular to the vertically polarized exciting light, respectively, and G, the instrumental correction factor which is ideally 1, is defined as $G = I_{HH}/I_{HV}$.

Time-Resolved Phosphorescence Anisotropy Measurements. Prior to phosphorescence measurements, samples were deoxygenated by using the oxygen-consuming enzyme system described by Horie and Vanderkooi (1981). Approximately 300 μL of reconstituted DSA at a concentration of 0.8–1.0 μM in a buffer containing 0.3% glucose was placed in a 5-mm square quartz glass cuvette (NSG Precision Cells, Inc.). After addition of glucose oxidase (0.072 mg/mL) and catalase (0.012 mg/mL) from stock solutions of 3 mg/mL, the cuvette was sealed with a rubber septum and parafilm. Argon was blown over the top of the sample, and after a 20-min incubation with the oxygen-consuming enzymes, deoxygenation was complete. To prevent oxygen leakage, a continuous, gentle flow of argon was maintained over the sample which also was gently stirred throughout the measurement.

Phosphorescence measurements were made with an Edinburgh Instruments Model 199 fluorescence time-domain spectrometer. The desired sample temperature was maintained by circulating a coolant through the sample cell block. Samples were equilibrated at the desired temperature for at least 1 h prior to starting the measurement. In determinations of the effects of substrates, measurements were made on the identical sample before and after substrate addition.

To initiate ATP synthesis during measurements of the phosphorescence of the bR–DSA-coreconstituted system, the sample was illuminated with a Kodak 600 H slide projector with an ELH lamp (300 W) focused on the sample cuvette side opposite to the excitation pulse. A glass cutoff filter (Corning 4-76) was placed between the projector light and the sample to reduce long-wavelength (>600 nm) light. In addition, to reduce the total light intensity of the actinic source by 85%, calibrated neutral density filters (Ditric Optics) and a Bentham Model 218 optical chopper were used. These conditions were necessary to prevent light scattering caused by the intense and continuous illumination of the sample from interfering with the detection of phosphorescence. When bR–DSA vesicle preparations were assayed for ATP synthesis activity under these conditions, rates were low [~1.4 nmol/(mg·min)], but above background levels [~0.4 nmol/(mg·min) in the dark], indicating that a pH gradient had formed. The typical synthetic activity of these vesicles at high light intensities is a few hundred nanomoles per milligram per minute (Krupinski & Hammes, 1986).

Sample excitation was achieved by using a nitrogen laser-pumped dye laser (PRA International Nitrodye LN120C) at a repetition rate of 10 Hz, providing a 10-μJ pulse with a duration of 0.3 ns. The broad-band dye laser emission using 10 mM coumarin (2A500, PRA International) in ethanol was from 500 to 530 nm. The phosphorescence emission was detected with a red-sensitive photomultiplier (Edinburgh Instruments) through a Schott RG-665 filter (Ealing Optics). The signal was averaged and digitized with an Ortec-Norland multichannel analyzer (Model 5500, minimum sampling interval 10 μs/point) and transferred to an AST computer for analysis. Data analysis software was from Edinburgh Instruments.

For determinations of emission anisotropy, an excitation polarizer was placed in the vertical position, and sequential measurements of the vertically polarized emission, $I_{VV}(t)$, and the horizontally polarized emission, $I_{VH}(t)$, were made, rotating the emission polarizer between the vertical and horizontal positions every 2000–4000 laser flashes. Data were collected for 20 000–48 000 flashes in each orientation per sample. Blank corrections were made by subtracting data acquired under identical conditions with unlabeled DSA. In addition, an instrumental correction factor (G factor, as defined above) to account for differences in the static sensitivities of the instrument in the two polarizer orientations was determined.

Data Analysis. After subtraction of background signals from vertical and horizontal data sets, phosphorescence anisotropy, $r(t)$, was calculated according to the equation:

$$r(t) = \frac{GI_{VV}(t) - I_{VH}(t)}{GI_{VV}(t) + 2I_{VH}(t)}$$

Nonlinear least-squares analysis then was used to fit the time-dependent anisotropy to one exponential and a constant:

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-t/\phi)$$

where ϕ is the rotational correlation time, r_0 is the anisotropy at the initial time of measurement, and r_∞ is the anisotropy at long times. Goodness of fit was determined by minimization of χ^2 where

$$\chi^2 = \sum_{\text{data}}\left[\frac{r(i) - F_D(i)}{\sigma(i)}\right]^2$$

Here the $r(i)$ values are the decay data, the $F_D(i)$ values are the fitted functions, and the $\sigma(i)$ values are the standard deviations of $r(i)$. The weighted residuals, $R(i)$, also were calculated and are defined as

$$R(i) = \frac{r(i) - F_D(i)}{\sigma(i)}$$

Results

Labeling of DSA with ErITC resulted in a stoichiometry of 0.2–0.5 mol of ErITC per mole of DSA with >90% of the label on the γ-polypeptide of CF_1. The presence of ErITC did not inhibit the ATP–P_i exchange activity of the reconstituted enzyme. Furthermore, exposure of the reconstituted enzyme to the laser flash for several hours during anisotropy determinations had a negligible effect on exchange activity.

The decay of total phosphorescence emission ($I_{VV} + 2I_{VH}$) of ErITC free in aqueous solution is shown in Figure 1A. The two triplet-state lifetimes obtained from this measurement are 32 ± 2 μs (27%) and 205 ± 3 μs (73%), where the relative amplitudes of the decay are given in parentheses. As expected, the very small anisotropy (0.009) of ErITC in solution did not decay with time. The decay of total phosphorescence intensity at 4 °C of ErITC–DSA reconstituted into phospholipid vesicles (Figure 1B) requires at least three exponentials for an adequate fit. The lifetime components obtained are 48 ± 0.3 μs (27%), 145 ± 3 μs (43%), and 508 ± 7 μs (30%). Such multiexponential decays are typical of protein-conjugated probes (Austin et al., 1979; Bartholdi et al., 1981; Zidovetzki et al., 1986;

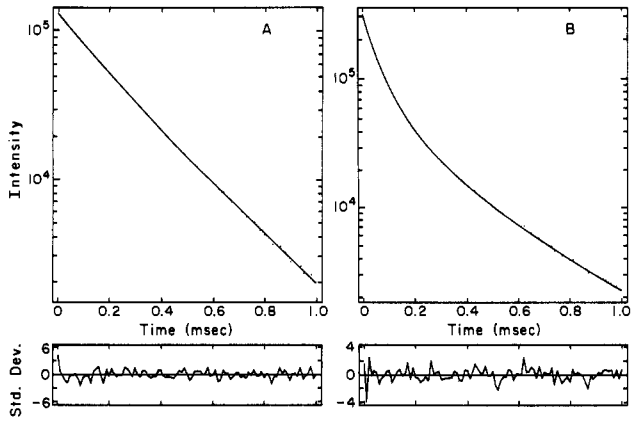

FIGURE 1: Total phosphorescence emission intensity versus time at 4 °C. Data were collected at 10 μs per channel. (A) Phosphorescence of free ErITC in 150 mM KCl, 2 mM EDTA, and 10 mM Na-Tricine, pH 8.0. (B) Phosphorescence of ErITC–DSA reconstituted into phospholipid vesicles in 150 mM KCl, 2 mM EDTA, 10 mM Na-Tricine, and 30% sucrose, pH 8.0. The solid lines are nonlinear least-squares fits of the data to two (A) and three (B) exponentials with the best-fit parameters given in the text. The residuals for each fit are shown in the lower plots.

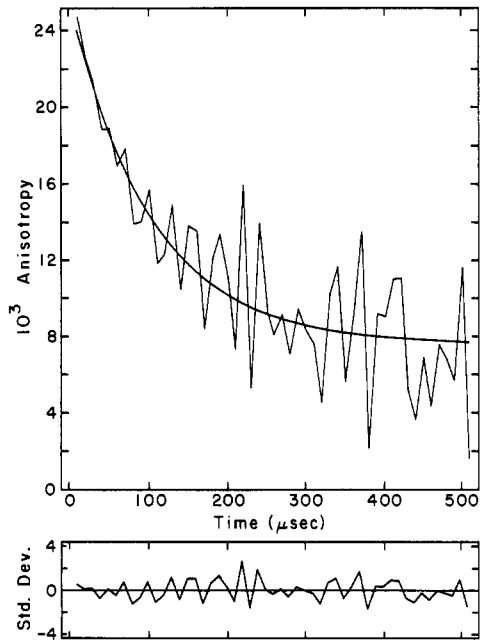

FIGURE 2: Phosphorescence emission anisotropy versus time at 4 °C of ErITC-labeled DSA reconstituted into phospholipid vesicles. Data were collected at 10 μs per channel. The vesicles were in 150 mM KCl, 2 mM EDTA, 10 mM Na-Tricine, 30% sucrose, 0.3% glucose, 3 mM Mg^{2+}, and 3 mM ATP, pH 8.0. The solid line represents the nonlinear least-squares fit to a single exponential plus a constant. The rotational correlation time obtained is 102 ± 17 μs. The residuals for this fit are shown in the lower plot.

Ludescher & Thomas, 1988).

A typical example of the time dependence of the phosphorescence anisotropy of ErITC–DSA reconstituted into phospholipid vesicles is shown in Figure 2. The instrumental correction factor (G factor) used to calculate anisotropy decays was 1.02. The decay parameters obtained from single-exponential fits of the experimental anisotropy curves under a variety of conditions are summarized in Table I. These parameters describe the anisotropy behavior of ErITC–DSA in the presence and absence of substrate at different temperatures, protein:lipid ratios, external medium viscosities, and membrane viscosities. The initial anisotropy, r_0, in all of these decays (Table I) is much smaller than the theoretical maximum value (0.4) or the value for erythrosin in a plastic (0.25)

Table I: Phosphorescence Anisotropy Decay Parameters of Membrane-Reconstituted ErITC–DSA

conditions[a]	anisotropy parameters[b] ϕ (μs)	r_0	r_∞	r_∞/r_0
no substrates[c]	176 ± 55	0.025	0.010	0.40
ADP + P_i[d]	177 ± 60	0.022	0.011	0.50
ATP (3 mM)[d]	117 ± 21	0.025	0.009	0.36
4 °C	215 ± 67	0.023	0.006	0.26
10 °C	135 ± 36	0.026	0.010	0.38
30 °C	60 ± 27	0.028	0.014	0.50
0% glycerol[e]	104 ± 29	0.019	0.006	0.32
50% glycerol[e]	93 ± 16	0.034	0.018	0.53
0 mol % cholesterol[f]	178 ± 23	0.029	0.006	0.21
17 mol % cholesterol[f]	221 ± 37	0.035	0.016	0.46
32 mol % cholesterol[f]	559 ± 151	0.059	0.042	0.71
2.5 mg/mL asolectin	120 ± 25	0.059	0.027	0.46
5.0 mg/mL asolectin	98 ± 49	0.039	0.025	0.64
7.5 mg/mL asolectin	74 ± 21	0.045	0.027	0.60

[a] Experiments were performed at 4 °C in 150 mM KCl, 2 mM EDTA, and 10 mM Na-Tricine, pH 8.0, with 0.8–1.0 μM DSA, 0.3% glucose, 3 mM Mg^{2+}, 30% sucrose, 5 mg/mL asolectin, 3 mM ADP, and 3 mM P_i, unless otherwise indicated. [b] Parameters are from single-exponential fits of anisotropy data using the first 500 μs with the first time point 10 μs after the laser flash. ϕ is the rotational correlation time, r_0 is the initial anisotropy, and r_∞ is the anisotropy at $t = \infty$. [c] Average of four experiments performed without ADP, ATP, or P_i. [d] Average of two experiments; without ADP and P_i when ATP is present. [e] No sucrose present. [f] Data were analyzed over 1 ms.

(Jovin et al., 1981). The anisotropy decays to a smaller but, in most cases, nonzero value (r_∞).

Anisotropy measurements with reconstituted ErITC–DSA in the absence of sucrose (data not shown) resulted in anisotropy decays characterized by a small amplitude (<0.01) and a flattening of the decay curve (increase in r_∞) with time. Consequently, variable results for the rotational correlation time, ϕ, especially in the absence of substrates, were obtained. The presence of 30% sucrose in the external medium, however, stabilized the vesicle–DSA preparation and resulted in larger amplitude anisotropy decays ($r_0 - r_\infty$ = 0.011–0.032) and reproducible rotational correlation times. The rotational correlation time of ErITC–DSA reconstituted into phospholipid vesicles and measured at 4 °C under stabilizing conditions was found to be the same in the presence and absence of substrates (Table I): ϕ = 176 ± 55 μs in the absence of substrates, ϕ = 177 ± 60 μs in the presence of 3 mM ADP and 3 mM P_i, and ϕ = 117 ± 21 μs in the presence of 3 mM ATP.

An increase in temperature led to a shortening of the rotational correlation time (Table I, Figure 3). An activation energy of 8.4 kcal/mol was calculated from the slope of the plot of ln $(1/\phi)$ vs $1/T$ (Figure 3). From a second set of data (not shown), an activation energy of 10.6 kcal/mol was obtained. These results agree with the trends predicted from measurements of steady-state fluorescence anisotropy of DPH-labeled phospholipid vesicles. At 4 °C, the steady-state anisotropy, r, was 0.18, while at 25 °C, r = 0.12, a decrease of 33%.

To ensure that the measured rotational correlation times are not a reflection of the rate of vesicle rotation, the external medium viscosity was increased by including 50% glycerol in the vesicle buffer. The rate of vesicle tumbling is expected to be significantly reduced under these conditions. Since similar rotational correlation times were obtained in the presence or absence of sucrose or glycerol (Table I), vesicle tumbling is not being measured. The value of r_0 in the absence of sucrose or glycerol is relatively small (0.008–0.013) due to the instability (probably aggregation) of the vesicles. Some base-line drift also may occur under these conditions; however,

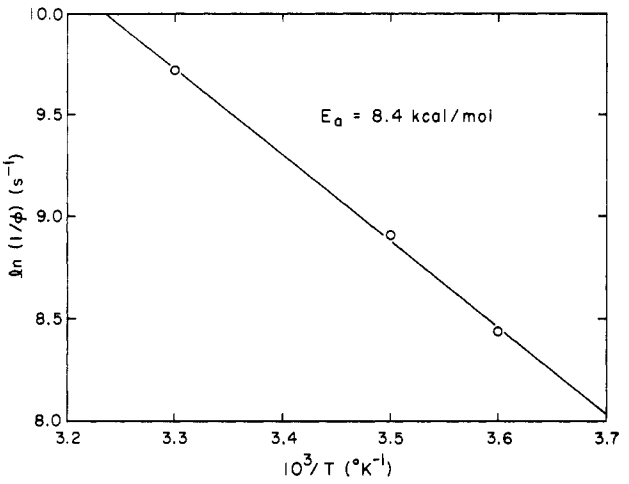

FIGURE 3: Temperature dependence of the rotational correlation time of ErITC-labeled DSA reconstituted into phospholipid vesicles. The rotational correlation times, ϕ, were determined at 4, 10, and 30 °C in 150 mM KCl, 2 mM EDTA, 10 mM Na-Tricine, 30% sucrose, 3 mM Mg^{2+}, 0.3% glucose, 3 mM ADP, and 3 mM P_i, pH 8.0. The slope of the straight line fit through the experimental points was used to calculate the activation energy for rotational diffusion, E_a.

the rotational correlation time is not significantly different in repetitive experiments.

Cholesterol incorporation into phospholipid vesicles was used to assess the effects of changes in membrane viscosity on the rotational correlation time. The effects of cholesterol are dependent on temperature and lipid composition, and its presence has been shown to increase the microviscosity of the membrane (Shinitzky & Inbar, 1974; Cooper et al., 1978; Muhlebach & Cherry, 1982). The specific effect of cholesterol incorporation into the asolectin vesicles used in these experiments was determined by steady-state fluorescence anisotropy measurements of DPH-labeled cholesterol/asolectin vesicles. Measurements at 4 °C indicate that the steady-state anisotropy increased by 27–39% in the presence of 26–37 mol % cholesterol. The increase in the steady-state anisotropy was even greater at 25 °C (66–92%). The ATP–P_i exchange activity of DSA reconstituted into vesicles with and without 25 mol % cholesterol also was measured. A 90% loss in exchange activity was observed with the cholesterol/asolectin reconstitution. An increase in the mole percent cholesterol in the phospholipid vesicle preparation significantly increases the rotational correlation times (Table I). The time-resolved results, therefore, agree with those from the steady-state measurements.

The rotational correlation time also was measured at different lipid:protein ratios. At 2.5 mg/mL asolectin, corresponding to an approximate lipid:protein ratio of 3700, ϕ was 120 ± 24 μs. A 3-fold increase in asolectin concentration resulted in a small reduction in ϕ (74 ± 21 μs).

The rotational correlation time of ErITC–DSA coreconstituted with bR was unaffected by the addition of ADP and P_i (Table II). The presence of light which initiates proton gradient formation and, in the presence of ADP and P_i, ATP synthesis also had little effect on the rotational correlation time.

The quantity r_∞/r_0 is listed in the last column of Tables I and II. This ratio, a measure of the "degree of orientational constraint" (Kinosite et al., 1977, 1984; Szabo, 1984; Zidovetzki et al., 1986), varies slightly with temperature, external medium viscosity, and membrane viscosity, and when the bR–DSA system is in the presence of light.

The values of the rotational correlation times were quite reproducible for a given DSA preparation and reconstitution into phospholipid vesicles. For different preparations of DSA, however, the rotational correlation times sometimes varied significantly (±50%), and for this reason, all comparisons are made between samples prepared on the same day.

Table II: Phosphorescence Anisotropy Decay Parameters of Membrane-Reconstituted bR–ErITC–DSA

conditions[a]		anisotropy parameters[b]			
light[c]	MgADP + P_i	ϕ (μs)	r_0	r_∞	r_∞/r_0
−	−	119 ± 17	0.051	0.024	0.47
+	−	101 ± 21	0.036	0.005	0.15
−	+	103 ± 19	0.049	0.023	0.47
+	+	158 ± 55	0.034	0.005	0.13

[a] All measurements were performed on the same sample at 10 °C in 150 mM KCl, 2 mM EDTA, 10 mM Na-Tricine (pH 8.0), 0.8 μM DSA, 2.5 mg/mL asolectin, 3 mM Mg^{2+}, 30% sucrose, 45 mM glucose, and 10 units/mL hexokinase with (+) and without (−) 4 mM MgADP and 10 mM P_i. [b] Parameters are from single-exponential fits of anisotropy data using the first 500 μs with the first time point 10 μs after the laser pulse. ϕ is the rotational correlation time, r_0 is the initial anisotropy, and r_∞ is the anisotropy at $t = \infty$. [c] Light intensity (+) sufficient for proton gradient formation. Rates of ATP synthesis were ~1.4 nmol/(mg·min).

Discussion

The anisotropy decay of ErITC-labeled DSA and bR–DSA reconstituted into phospholipid vesicles has been measured under a variety of conditions. The lipid:protein ratio was kept sufficiently high to avoid significant interactions between protein molecules. Moreover, the lipid phase transition for asolectin vesicles is below zero (Ladbrooke & Chapman, 1969). The choice of ErITC as a phosphorescent probe for this study was based on its high quantum yield for triplet formation (98%) and negligible prompt fluorescence (<2%) (Thomas, 1986). Specific labeling conditions were achieved such that primarily the γ-polypeptide of CF_1 was labeled with negligible effects on ATP–P_i exchange activity.

Low absolute values of the initial anisotropy, r_0, such as those observed in these experiments, are a result of rotational motion on a time scale too fast to be detected (Burkli & Cherry, 1981). Rapid motion (<10 μs) could be due to any combination of the following: (1) independent motion of the phosphorescent probe; (2) segmental motion of part of the protein containing the probe; and (3) rotation of the protein in the membrane. The observed nonzero value of the limiting anisotropy, r_∞, is not an unusual property of integral membrane proteins (Zidovetzki et al., 1986) and implies that the motion of the probe is somewhat restricted.

Over the period of a typical anisotropy determination (2–4 h) and in the absence of sucrose, the time dependence of the anisotropy is observed to change. The amplitude of the decay decreases and the value of r_∞ increases. Similar effects have been seen with other systems (Restall et al., 1981; Zidovetzki et al., 1981). This change, indicative of slower protein movement, can be attributed to a decrease in lipid fluidity, protein aggregation, or vesicle aggregation. Fortunately, the addition of 30% sucrose to the external medium has a stabilizing effect on the DSA–vesicle preparation, and allowed the dependence of ϕ on temperature and the presence of substrates to be determined. Instrumental artifacts that interfered with the first 10 μs after the laser flash, coupled with the instrument resolution of 10 μs/point, prevented the measurement of anisotropy decay occurring at times less than 10 μs.

The theoretical values for the rotational correlation times of a rigid cylinder undergoing uniaxial rotation about an axis normal to the plane of the membrane are ϕ and $\phi/4$, where $\phi = 4V\eta/kT$ (Cherry, 1978; Thomas, 1986). In this equation, k is Boltzmann's constant, T is the absolute temperature, and V is the volume of the enzyme imbedded in the membrane and

experiencing the membrane microviscosity η (Saffman & Delbruck, 1975). Assuming the value of η to be 4 P (Shinitzky & Inbar, 1974), a molecular weight of 100000 for the portion of the DSA (CF_0) in the membrane, and a partial specific volume of 0.73 cm^3/g (Cantor & Schimmel, 1980), the theoretical rotational correlation times at 4 °C are in the range 13–51 μs. This calculation does not take into account the external portion of the DSA (CF_1), which has a molecular weight of 400000, or the enzyme's complex shape. These factors should slow down the rate of DSA rotation. Therefore, the calculated values are in reasonable agreement with the measured rotational correlation times. The precision of the data is insufficient to resolve the expected multiple rotational correlation times.

The lack of dependence of the rotational correlation time on the medium viscosity and its dependence on cholesterol indicate that rotation of the enzyme in the phospholipid vesicles is being measured. Moreover, the activation energy of ϕ is approximately that expected for rotational diffusion in a membrane (Shinitzky & Inbar, 1974; Bigelow et al., 1986). The phosphorescent probe is bound to CF_1, which is not in the membrane. Since the rotation measured is characteristic of rotation in a membrane, CF_1 and CF_0 must be tightly coupled; that is, CF_1 and CF_0 are not rotating relative to each other on this time scale.

Rotational correlation times for both DSA and bR–DSA vesicle preparations did not change significantly upon the addition of substrates or during ATP synthesis. Since the enzyme rotates as a single unit under the experimental conditions employed and no significant change in the phosphorescence anisotropy decay was detected upon addition of substrates, formation of a pH gradient, or ATP synthesis, rotation of the enzyme molecule does not appear to play a significant role in enzyme catalysis or ion pumping. Although the rate of molecular rotation is much greater than the turnover number for ATP synthesis (even under optimal conditions), some change in molecular rotation might be anticipated if rotation were involved in the catalytic process. These results do not exclude the possibility that slower rotations of the molecule (or within the molecule) than measured here are important for function.

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