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Fast rotation of EITC-labelled proteolipid after reconstitution of chloroplast ATP synthase in bilayer membranes

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The chloroplast ATP synthase (CF_0CF_1) has been isolated and labelled with the triplet probe eosin isothiocyanate (EITC) when dissolved in detergent solution. On SDS-PAGE gels of the labelled enzyme, eosin fluorescence was observed almost exclusively in a single band with a molecular mass of about 8000 Da, subunit III of CF_0. At saturation, about 10 mol EITC / mol CF_0CF_1 were bound, which may indicate, that the complex contains 10 copies of subunit III. The CF_0CF_1 complex labelled in subunit III was incorporated into liposomes using the dialysis technique and the enzyme rotational diffusion in the liposome membrane was measured using laser-flash-induced absorption anisotropy of the protein-bound EITC. At 30 °C and a lipid / protein ratio of $8 \cdot 10^4$: 1 mol / mol uniaxial rotation with a correlation time of 200 ns at 30 °C was observed. Considering the model of Saffman and Delbrück (Saffman, P.G. and Delbrück, M. (1975) Proc. Natl. Acad. Sci. USA 72, 3111–3113) and the viscosity of the azolectin membrane (Wagner et al. (1989) Eur. J. Biochem. 182, 165–173), this rotational correlation time corresponds to uniaxial rotation of a protein cylinder in the membrane with the radius of 1.1 nm. The rotating unit with this dimensions can easily be formed by two transmembrane helices. We therefore conclude that the proteolipid oligomer in CF_0, probably due to binding of EITC at lysine-48, dissociated into monomers. The other subunits of CF_0CF_1 apparently remained stable parts of the ATP synthase complex and associated with the liposome membrane. In a second attempt, CF_0CF_1 was labelled with EITC when bound to the thylakoid membrane. In this case, on SDS-PAGE gels of the labelled enzyme, eosin fluorescence was observed in a single band with a molecular mass of about 57 kDa, corresponding to the β subunit of CF_1. After incorporation of the β-labelled CF_0CF_1 into liposomes the rotational correlation time of the enzyme was 1.3–1.5 μs at 30 °C. This rotation time is compatible with the uniaxial rotation of a 'protein cylinder' in the membrane with the diameter of 6.1 nm. Obviously, this rotating unit represents the entire CF_0CF_1 complex. The rotation rate of the reconstituted enzyme complex appeared to be determined mainly by the friction experienced by CF_0 within the membrane and therefore the observed 'rotating unit' represents almost exclusively CF_0. Addition of 2 μM venturicidin or 2 μM tributyltin, which are known to block proton flow through CF_0, to proteoliposomes containing the β-subunit-labelled CF_0CF_1 increased the rotational correlation time of the enzyme to about 2 μs. This result shows that the both inhibitors block the H^+ flow through CF_0CF_1 by changing the overall structure of the F_0 part. In contrast to this result, with proteoliposomes containing the subunit-III-labelled CF_0CF_1 no changes in the rotational time were observed after addition of venturicidin and tributyltin. This indicates that binding of either of the two inhibitors does not affect the conformation of the monomeric subunit III to any remarkable extent.

Introduction

Photophosphorylation in the thylakoid membranes of green plants is catalyzed by the oligomeric ATP synthase complex (CF_0CF_1). This enzyme complex consists of two separable moieties, a hydrophobic membrane part, CF_0, and a water-soluble part, CF_1. The membrane sector acts as an proton channel, whereas the

Abbreviations: EITC, eosin-5-isothiocyanate; CF_0, membrane part of the ATP synthase (proton channel); CF_1, soluble part of the ATP synthase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; $Pam_2GroPEtn$, L-α-dipalmitoylphosphatidylethanolamine.

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water-soluble CF_1 complex carries the catalytic site(s) (for reviews see Refs. 1, 2). Similar numbers of subunits are found in the two moieties of the homologous proton translocating F_0F_1 ATP synthases in mitochondria, bacteria and chloroplasts [1,2,9]. CF_1 is composed of five different subunits, α (59 kDa), β (56 kDa), γ (37 kDa), δ (17.5 kDa), ε (13 kDa) with a stoichiometry of 3 : 3 : 1 : 1 : 1. The catalytic sites of ATP synthesis are located in the β-subunits or between the alternating $\alpha\beta$ subunits [3,4].

The hydrophobic CF_0 entity contains four different subunits, I (18 kDa), II (16 kDa), III (8 kDa) and IV (25 kDa). The stoichiometry of these subunits is still under debate, but is proposed to be most likely 1–2 : 1–2 : 6–12–1 (for reviews see Refs. 9, 10, 13). In detail, little is known about the functional role of the individual subunits, especially in the entire CF_0 part of the complex. Subunits IV and I from CF_0 are homologous to the *Escherichia coli* subunits a and b, which were supposed to be involved in binding of EF_1 to the membrane. Subunit III, which is usually called 'proteolipid', contains a DCCD binding site and seems to play an essential role in forming a channel structure capable specifically of conducting protons across the membrane [11–13]. From secondary structure predictions, a hairpin structure with two membrane-spanning helices and a hydrophilic loop facing the F_1 binding site has been proposed for the homologous proteolipids from different sources [12,13].

It is not known how proton flow is coupled to ATP synthesis. Two main concepts are discussed in the literature: (i) 'direct coupling': the translocated protons are directly channeled to the catalytic sites of CF_1, thus providing directly energy required for catalysis; (ii) 'indirect coupling': the translocated protons drive conformational changes of subunit(s) and the energy stored in protein conformation is used in ATP synthesis [1,2,9]. At present, the later concept seems to be favoured by most of the workers in the field [9,14]. Evidence for long-range conformational interactions between F_0 and F_1 has been presented for the mitochondrial enzyme [15,16].

Models for conformational coupling have been worked out explicity [17–19] and it was hypothesized that, analogous to the flagellar rotor in bacteria [20], rotational motion of subunits might drive ATP synthesis. However, up to now no direct evidence has been presented for the 'rotational catalysis' (for a recent review on this issue, see Ref. 9).

We were aiming to obtain information on the topological organization of the membrane part of the chloroplast ATP synthase. For this we investigated the rotational mobility of EITC-labelled CF_0CF_1, which was reconstituted into liposomes. Studies on the translational and rotational diffusion of membrane proteins indicated that, in reconstituted systems in the liquid-crystalline state, rapid diffusion of these proteins occurred [21–23,49]. The rate of rotational motion can be used to deduce information on the size of the 'rotating unit', provided the dynamic friction opposing the motion is known. We have previously characterized the dynamic friction in the membrane of azolectin liposomes by measuring the rotational diffusion of EITC-labelled lipid molecules [23]. Here we demonstrate that, by combining the results of CF_0CF_1 rotational diffusion and lipid rotation, we are able to obtain information of that part of the ATP synthase complex which is embedded in the liposome membrane.

Our results show that the CF_0 oligomer is rather unstable, since the proteolipid, after chemical modification, dissociated into the monomeric form. However, after dissociation of the proteolipid, the remaining parts of the ATP synthase appeared to be still bound to the liposome membrane.

Materials and Methods

CF_0CF_1 was purified from spinach thylakoids as described in Ref. [24]. When labelling was performed on thylakoid membrane bound CF_0CF_1, the purification protocol was changed as follows. After the last centrifugation step of the thylakoid preparation and before solubilization of the CF_0CF_1 complex, the pellet was resuspended in 100 ml of a buffer with 20 mM Na-Tricine (pH 8.0)/400 mM sucrose/5 mM $MgCl_2$ at a chlorophyll concentration of 4 mM. EITC (2 mg/ml) was solubilized in the same buffer and aliquots of this solution were added to the thylakoid suspension to give the desired final EITC concentrations. After 10 min incubation the reaction was stopped with 1 ml 1 M glycine. The thylakoids were centrifuged (13 000 × g, 15 min) and washed in the same buffer and again resuspended in the above buffer. To remove unbound EITC, the labelled thylakoids were then passed through a Sephadex G-25 column (20 × 1.5 cm) equilibrated with the above buffer. Further purification steps were then performed as described [24]. Binding of EITC to CF_0CF_1 was also followed after EDTA extraction of labelled CF_1 from the thylakoid membrane using a microscale extraction procedure described by Cerrione et al. [25]. Since the fluorograms of CF_0CF_1 labelled in the thylakoid membrane showed that no membrane components were labelled using the above protocol, labelling of the ATP synthase was then followed routinely by extraction of labelled CF_1 and subsequent determination of the amount of bound EITC.

In a second attempt, labelling was performed after partial purification of the enzyme. The ammonium sulphate precipitate fraction containing about 10% CF_0CF_1 was resuspended in 20 mM Na-Tricine (pH 8.0)/200 mM sucrose/5 mM $MgCl_2$/0.2 mM ATP

buffer to a protein concentration of about 10 mg/ml. This solution (0.5 ml) was incubated 15 min with 0.5 ml of the same buffer, containing 0.2 to 2.4 mg EITC/ ml. The reaction was terminated with a 10-fold excess of glycine and the unbound EITC was removed by gel-filtration on Pharmacia PD-10 columns equilibrated with 30 mM Tris-succinate (pH 6.5)/0.2 mM ATP/0.5 mM EDTA/2 $g \cdot l^{-1}$ Triton-X 100. The column eluate containing the labelled CF_0CF_1 was then directly used in the following sucrose density gradient centrifugation [24].

Protein was determined according to Sedmak and Grossberg [26] and the stoichiometry of the bound eosin was determined spectrometrically with a Hitachi 150-20 spectrophotometer using an absorption coefficient of $8.35 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for the dye [27].

Incorporation of the purified CF_0CF_1 into azolectin liposomes and determination of the ATP synthesis yield was followed by the luciferin/luciferase assay after an acid/base transition as described [24].

The principles and instrumentation of measuring the rotational diffusion of proteins with extrinsic triplet probes under photoselection are given in detail elsewhere [43,44]. Briefly, the eosin absorption changes at 502 nm due to ground-state depletion and its subsequent repopulation from the triplet state were used for measurement. This wavelength was selected because here the flash burst of the laser was minimal. Excitation of the eosin absorption changes normal to the measuring light flash was provided from a intrinsically polarized frequency-double Nd-YAG laser (J.K. Asers) at 532 nm with a flash duration of 5 ns (fwhm). Homogeneous excitation of the samples (3 ml, 1 cm optical path) was achieved by passing the excitation flash through a beam expander. The energy of the laser was adjusted to saturate no more than 30% of the eosin absorption changes (typically 0.2–0.5 mJ/cm^2). As measuring light source, a xenon flash lamp with tunable duration (50–000 μs) and an energy of about 1 J/s was used. The actual light intensity applied to the sample after passage of the light through a wide band filter (500–520 nm) and a sheet polarizer (Schott) was 0.2–0.5 J/s. For detection, a photodiode (FND 100, Polytec) with 0.5 cm^2 active area and a rise time (C_{90}) of 0.7 ns was used. The signal from the photodiode was amplified (100-times) and recorded on a Biomation 6500 transient recorder digitally interfaced to a Tracor TN 1500 averaging computer. The time resolution of the instrumental arrangement was 2 ns/address, but it was limited to times lower than 30 ns because of the unavoidable flash burst. The d.c. output of the photodiodide and the energy of the excitation flash were both monitored and averaged in each experiment.

The absorption changes $\Delta A_{\parallel}(t)$ and $\Delta A_{\perp}(t)$ for parallel and perpendicular polarisation between the *E*-vector of the excitation and measuring light were combined to yield the absorption anisotropy:

$$r(t) = \Delta\Delta A(t)/\Delta A_{tot}(t) \tag{1}$$

with the total absorption change:

$$\Delta A_{tot}(t) = \Delta A_{\parallel}(t) + 2\Delta A_{\perp}(t) \tag{2}$$

and the difference of the absorption changes:

$$\Delta\Delta A(t) = \Delta A_{\parallel}(t) - \Delta A_{\perp}(t) \tag{3}$$

To improve the signal-to-noise ratio in the measurements with reconstituted CF_0CF_1, 200 signals were typically averaged in succession in each experiment and further improvement was achieved by averaging the data from three independent measurements.

From the absorption changes $\Delta A_{\parallel}(t)$ and $\Delta A_{\perp}(t)$ the time-course of the absorption anisotropy was calculated according to Eqn. 1 on a PDP 11/34 computer. The anisotropy decay curves were then analysed for their exponential decay components by a computer program based on the Fourier convolution theorem according to Provencher [31]. The program fits noisy raw data by multiexponential decay curves without the need of initial guesses concerning the number of components or their decay constants. Although fitting of decay curves with more than one exponential is in general a difficult task, the above program provides a rather rigorous analysis of the experimental data [31,32].

In all experiments with the reconstituted ATP synthase it was not possible to obtain a good fit of the anisotropy decay curves with only one exponential. The best fit of the data was obtained according to the following expression:

$$r(t) = a_1 \cdot \exp(-t/\phi_1) + a_2 \cdot \exp(-t/\phi_2) + a_3 \tag{4}$$

Typical standard deviations of the fit parameters were less than 10% and that of the fit less than 10^{-3}. The analysis with the Provencher program provided only the mathematically best fit of the decay curves without imposing any physically meaningful model. The total absorption changes ($\Delta A_{tot}(t)$) typically decayed with a time constant of $\tau \simeq 80$ μs ($\leqslant 95\%$) and $\tau \simeq 0.6$ ms ($\geqslant 5\%$).

The theory of rotational diffusion of proteins and lipid components in membranes has been reviewed in detail [33,34]. The rotational diffusion of membrane proteins usually is described by the model of Saffmann and Delbrück [35], in which an intrinsic membrane protein is depicted as a cylinder with the radius r_a spanning the fluid membrane of the viscosity η_2. This model assumes that rotation occurs only about the axis normal to the membrane plane and the viscosity of the suspension medium, η_1, is much smaller than the membrane viscosity, η_2. The decay of the absorption ani-

sotropy in this case is described by [33]:

$$r(t) = a_1 \cdot \exp(-t/\phi) + a_2 \cdot \exp(-4t/\phi) + a_3 \quad (5)$$

where $\phi = D_{\parallel}^{-1}$ and $D_{\parallel}$ is the diffusion coefficient for the rotation about the axis normal to the membrane plane; $a_1 = 1.2 \cdot \sin^2\theta \cdot \cos^2\theta$; $a_2 = 0.3 \cdot \sin^4\theta$; $a_3 = 0.1 \cdot (3 \cdot \cos^2\theta - 1)^2$ where θ is the angle between the transition dipole moment of the chromophore and the membrane normal.

Results and Discussion

Preparation, labelling and reconstituted of CF_0CF_1

Preparation of the ATP synthase complex was performed as described in detail by Fromme et al [24]. With this procedure the entire CF_0CF_1 complex was obtained as a water-soluble micelle. Fig. 1, left lane, shows a SDS-PAGE gel after silver-staining of a typical CF_0CF_1 preparation. All subunits (except subunit IV) are clearly visible and no other contaminations were observed. Therefore, the preparation appeared to be essentially pure. Probably due to its low staining intensity, the band of subunit IV is not visible [24]. In order to prove the structural and functional integrity of the purified ATP synthase, it was reconstituted into liposomes using the dialysis technique [24,28] and the ATP synthesis driven by an artificially imposed ΔpH across the membrane was measured [28]. For different CF_0CF_1 preparations in liposomes containing about 0.3 mg protein/ml, we obtained an ATP yield between 38 and 46 ATP/CF_0CF_1 per s, which is in the same order of magnitude as observed by Schmidt and Gräber [28].

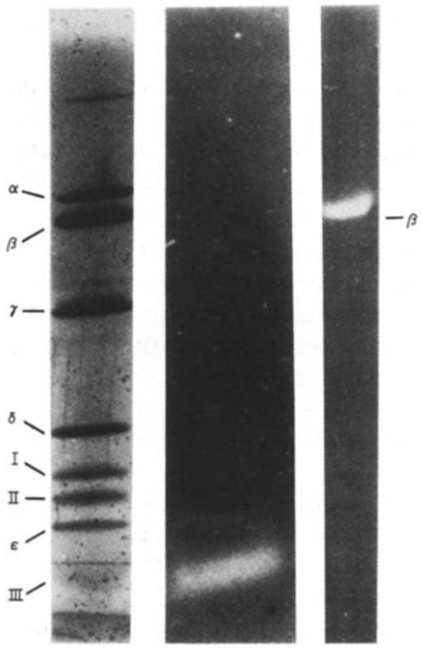

Fig. 1. Subunit distribution of the EITC-accessible lysine residue in CF_0CF_1. Left lane. SDS-PAGE of purified EITC labelled CF_0CF_1 with a 12% separating gel run on the Pharmacia Phast system. 0.1 μg CF_0CF_1 in 0.3 ml standard buffer (Pharmacia) were applied. EITC labelling was as in the middle lane. Silver-staining of the bands was performed as described [57]. The identity of the individual bands is indicated. Middle lane. Fluorescence gel of EITC-labelled CF_0CF_1. Labeling of the CF_0CF_1 detergent micelle was performed as described in Materials and Methods. 8 mol EITC were bound per mol CF_0CF_1. SDS-PAGE was carried out with a discontinuous buffer system according to Schägger, and Von Jago, G. [58]. After fixing, the gels were illuminated on an UV transilluminator and photographed as described by Nailin et al. [59]. Right lane. Fluorescence gel of EITC labelled CF_0CF_1. Labelling of CF_0CF_1 on the thylakoid membrane was performed as described in Materials and Methods. All other experimental procedures as described in the middle lane.

As previously described [23,29,30], we used EITC, covalently attached to different sites in CF_0CF_1, as a triplet probe for measuring the protein rotational diffusion via absorption anisotropy [23,29,33,43,44]. Labelling of the enzyme complex was performed in two different approaches:

(i) CF_0CF_1 was labelled after solubilization and partial purification of the enzyme. In this case, the crude CF_0CF_1 complex was labelled in the micelle and, after the labelling procedure, further purified to the final state.

(ii) CF_0CF_1 was labelled in thylakoid membranes.

In the following, we will first describe the results obtained for labelling in the micelle (case (i)). Fig. 2a shows the amount of EITC bound to CF_0CF_1 as a function of the incubation concentration. As is obvious from this figure, the binding of EITC to CF_0CF_1 followed a typical binding isotherm. Saturation of EITC binding was reached at about 10 mol EITC/mol CF_0CF_1. The labelled CF_0CF_1 complex was subjected to SDS gel electrophoresis and protein bands containing bound EITC were monitored by fluorography. When CF_0CF_1 was labelled in the micelle, almost exclusively subunit III, the proteolipid, was labelled (Fig. 1, middle lane). When noncovalently bound EITC was not removed carefully, some EITC fluorescence could also be detected in the front. In some experiments, when high concentrations of labelled protein were applied to SDS gel electrophoresis, a fluorescent band with very low intensity could be detected at the 58 kDa region of the gel. The labelling of subunit III, at first sight, was a surprising result, since the hydrophobic subunit III should be hardly accessible for the EITC label from the water phase. However, under the conditions applied, always and almost exclusively the proteolipid was labelled. Apparently, one part of the proteolipid in the CF_0CF_1 micelle must be easily accessible from the bulk phase. Moreover, this part of the proteolipid should contain a reactive lysine, which is the only amino acid (except the N-terminus) capable of forming a stable bond with EITC [36]. From the known primary structure of subunit III from the spinach ATP synthase [37], a hairpin-like secondary structure has been proposed analogous to the proteolipids from *E. coli* and mito-

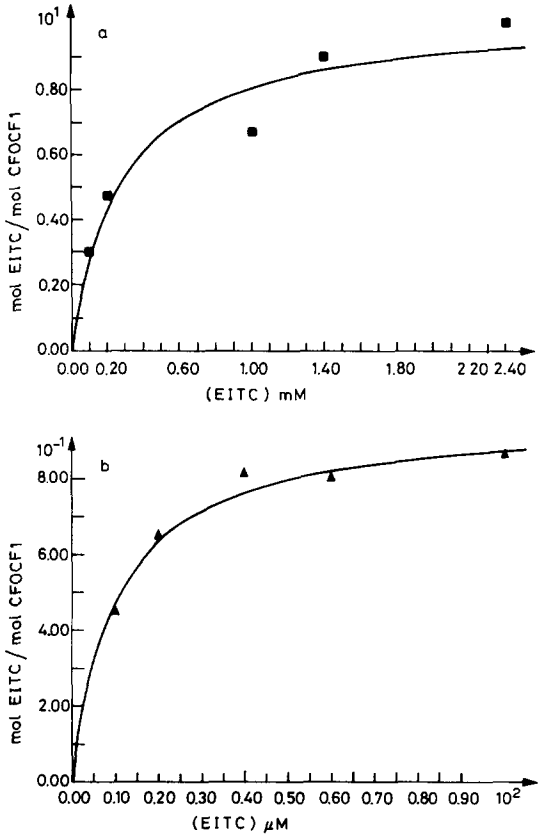

Fig. 2. Binding of EITC to CF_0CF_1. (a) Labelling of the CF_0CF_1 detergent micelle and determination of the amount of bound EITC was performed as described in Materials and Methods. Each point is the average of two independent determinations. (b) Labelling of the CF_0CF_1 on the thylakoid membrane and determination of the amount of bound EITC was determined by both isolation of the whole CF_0CF_1 and by EDTA extraction of CF_1 from thylakoids (see Materials and Methods). Each point is the average of two independent determinations.

chondria [13]. Considering this structure as very likely, EITC should be bound to lysine-48, the sole lysine in the proteolipid, which in addition is part of the hydrophilic turn structure between the two α helical structures [36].

The purified ATP synthase labelled in subunit III could also be reconstituted into liposomes using the dialysis technique. According to the following protocol, labelled and non-labelled enzyme were incorporated into the liposome membrane to the same extent. Two freshly prepared liposome samples containing labelled and nonlabelled CF_0CF_1 were passed through a Sepharose 4B column (55 × 16 mm) equilibrated with the dialysis buffer, which did not contain BSA. Then, the collected liposomes were centrifuged and the pellet resuspended in equal amounts of dialysis buffer (without BSA). In both samples protein and lipids were separated by acetone precipitation [38] and protein concentration was determined. The amount of recovered CF_0CF_1 was compared with the initial amount of protein applied for incorporation into the liposomes. For both samples, about 85% ($n = 2$, each sample) of CF_0CF_1 was recovered. This value for the incorporation of CF_0CF_1 into liposomes is in the same order of magnitude as observed by Schmidt [39]. Equal amounts of protein were then subjected to SDS gel electrophoresis. After silver-staining, all bands from the labelled and non-labelled ATP synthase as shown in Fig. 1, left lane, were visible and the intensity of the protein bands was practically identical for both samples.

Together, these results indicate that CF_0CF_1 labelled in subunit III is incorporated into the liposome membrane to the same extent and with the same subunit composition as the non-labelled enzyme. With liposomes containing CF_0CF_1 labelled in subunit III, even at a load of only 1 mol EITC/mol CF_0CF_1, ATP synthesis driven by an acid/base transition [28] was completely inhibited. Interestingly, the binding of EITC to subunit III of CF_0 seems to saturate at about 10 mol EITC/mol CF_0CF_1. Considering that binding of EITC on subunit III occurs only at a single site (Lys-48), this result would indicate that there exist 10 copies of subunit III/CF_0CF_1.

Fig. 2b shows the amount of EITC bound to CF_0CF_1, when labelling of the enzyme complex was performed in the thylakoid membrane. With the applied experimental protocol, 10 min of incubation period of the thylakoids and the rather low incubation concentration, binding of the dye seemed to saturate at about 1 mol EITC/mol CF_0CF_1. Fig. 1, right lane, shows the fluorography of the labelled CF_0CF_1 complex. As is obvious from this trace, when labelling was performed in the thylakoid membrane, the EITC fluorescence could be detected only in a band with molecular mass about 58 kDa, the β subunit of CF_0CF_1. As proved by the above-described experimental protocol, the ATP synthase labelled in the β subunit could also be incorporated into liposomes to the same extent and with the same subunit composition as the non-labelled enzyme. With these liposomes, containing the β-subunit-labelled CF_0CF_1, ATP synthesis still could be observed. The yield after an acid to base transition was between 20 and 30% of control liposomes containing the unlabelled enzyme.

The above results show, that depending on the label procedure, EITC was bound at different sites. When labelling of the enzyme complex was performed after solubilization and partial purification of the CF_0CF_1 micelle, EITC was preferentially bound to subunit III in CF_0. In contrast to this, when thylakoid membranes were incubated with EITC and CF_0CF_1 was subsequently isolated from these membranes, EITC was preferentially bound to the β subunit in CF_1. Similar results have been obtained previously with isolated CF_1, where EITC was preferentially bound to the β subunit [43]. The high reactivity of an R-NH_2 residue in subunit III in the CF_0CF_1 detergent micelle, however, was surprising, since, from known features of the ATP synthase

complex [1,2,9,13], the proteolipid should be hardly accessible for the label. Apparently the conformation of CF_0CF_1 in the detergent micelle is rather different from one in the membrane. We were not successful in labelling the proteolipid on thylakoid-membrane-bound CF_0CF_1, or, after reconstitution of CF_0CF_1, in the liposome membrane. The course of the EITC binding to the proteolipid in the CF_0CF_1 micelle indicates that a single class of binding sites is involved. This site is probably Lys-48, which was supposed to be in the more hydrophilic loop connecting the hydrophobic α-helical parts of subunit III [13,37]. The binding of a maximal amount of 10 mol EITC/mol CF_0CF_1 seems to indicate that CF_0CF_1 may contain 10 copies of the proteolipid. However, this figure has to be taken with caution, since it is based on protein determinations using BSA standards. For a more rigorous determination, amino-acid analysis would be required. The complete inactivation of the ATP synthesis in proteoliposomes by only about 1 mol subunit III bound EITC/mol CF_0CF_1 indicates that the proteolipid oligomer acts in a cooperative fashion, as proposed earlier [13]. Similarly, it has been shown that only a small fraction of the proteolipid has to be modified for a complete inactivation of F_0F_1 in bacteria [13].

Rotational diffusion of the CF_0CF_1 micelle and of CF_1 in solution

Fig. 3, trace a, shows the time-course of the absorption anisotropy of the subunit-III-labelled CF_0CF_1 micelle in solution and, for comparison, trace b, the time-course of EITC labelled CF_1 in solution. The rotational correlation time obtained for the subunit-III-labelled CF_0CF_1 micelle was 820 ns ($\eta = 1$ mPa · s, $T = 20\,^\circ$C). The micelle of CF_0CF_1 labelled in the β subunit revealed essentially the same rotational correlation time. These results show that the site of labelling does not affect the rotational diffusion of the detergent-protein complex in solution. Applying known formalisms for the interpretation of hydrodynamic data [40], from the above rotational correlation time a rough estimate of the longest distance in the protein detergent micelle may be obtained. Considering the shape of the ATP synthase complex [41] and taking into account that detergent binding will occur mainly on the F_0 part, assuming therefore that the bound detergent will have only a small effect on the overall rotation of CF_0CF_1, the spherical volume and diameter of the micelle can be calculated in a fair approximation from the measured rotational correlation time:

$$\tau = V_{\text{micelle}} \cdot \eta / kT \qquad (6)$$

From the calculated V_{micelle} the corresponding diameter of the CF_0CF_1 micelle can be calculated as $d = 18$ nm. The rotational diffusion of isolated CF_1 in solution has

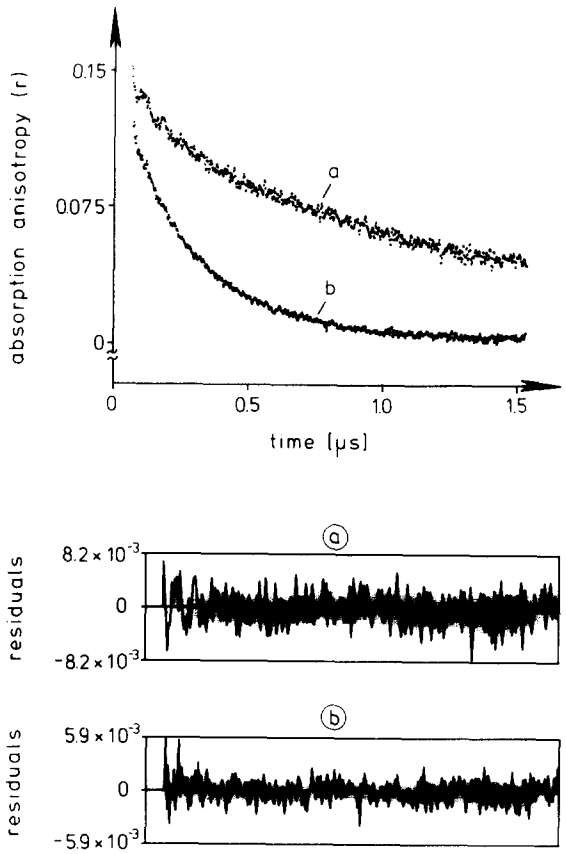

Fig. 3. Time-course of the absorption anisotropy for the EITC-labelled CF_0CF_1 detergent micel (trace a) and for EITC-labelled CF_1 (trace b) in solution. CF_0CF_1 was labelled in subunit III as described in Materials and Methods and CF_1 was isolated as in Refs. 43, 44. Eosin concentration in the measuring cuvette was 6 μM. Labelled CF_1 was contained in a buffer with 50 mM Tris-HCl (pH 8)/50 mM NaCl/5 mM $MgCl_2$. The shown traces were the average of 100 single laser flashes each. Measurements were performed at 20 °C at a time resolution of 2 ns/adress. By analysis of the decay curves the following parameters were obtained: trace (a) $r_0 = 0.17$, $\phi_1 = 820$ ns (72%), $\phi_2 = 74$ ns (28%); trace (b) $r_0 = 0.16$, $\phi_1 = 317$ ns (77%), $\phi_2 = 117$ ns (23%). In the lower part of the figure the residuals of the computer fit are shown.

been measured before [43,44]. Recent reinvestigators on this subject [44,45] showed in agreement with values published by Schinkel and Hammes [46] that the rotational correlation time of the isolated enzyme in a quite intact form was in the order of 320 ± 20 ns, while higher correlation times for the latent CF_1 seemed to indicate that some denaturation of the soluble enzyme had already occurred. Applying again the above formalism, we obtained for soluble CF_1 with $\tau_{\text{sph}} = 320$ ns a value of $d = 13$ nm. Aligning both axes and subtracting the axis of the water-soluble CF_1 from the axis of the CF_0CF_1 micelle, one obtains a length of about 5 nm, which should represent approximately the length to which the enzyme complex is embedded in the membrane.

Rotational diffusion of subunit-III-labelled CF_0CF_1 in the bilayer membrane

The ATP synthase complex labelled in subunit III was incorporated into liposomes using the dialysis technique and the rotational diffusion was measured. Fig. 4 trace b shows a typical curve obtained for the decay of the absorption anisotropy. The shown measurement was performed at 30 ° C and the proteoliposomes were contained in aequous buffer solution ($\eta \simeq 1$ mPa · s). In all measurements with subunit-III-labelled CF_0CF_1 in liposomes the decay of the absorption anisotropy could be fitted by only two exponentials according to Eqn. 4 (Materials and Methods section).

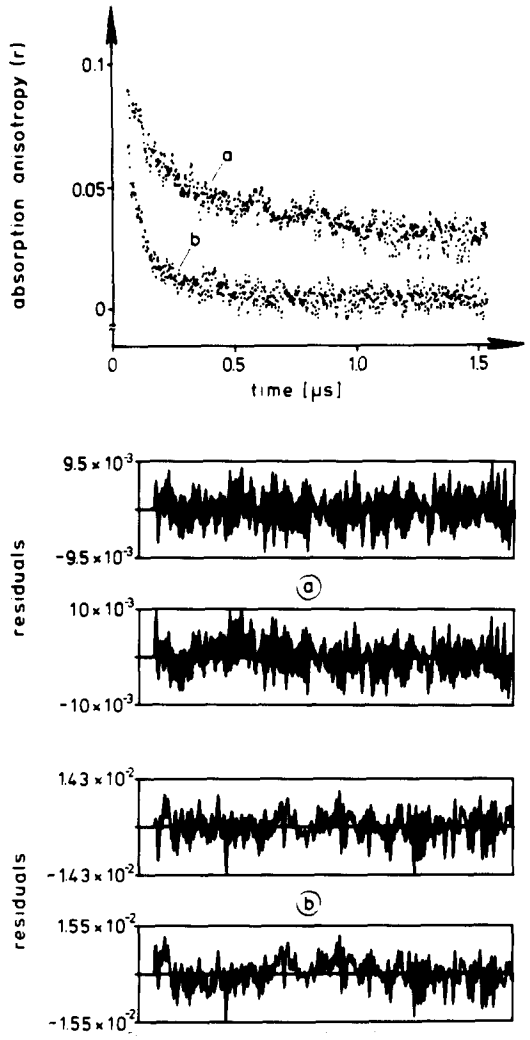

Fig. 4. Time-course of the absorption anisotropy for the EITC-labelled CF_0CF_1 reconstituted in liposomes. Trace b, liposomes containing subunit III labelled CF_0CF_1. Trace a, liposomes containing subunit β labelled CF_0CF_1. Both samples were contained in a buffer with 10 mM Tricine-NaOH/2.5 mM $MgCl_2$/0.25 mM dithiothreitol/0.2 mM EDTA. Measurements were performed at a time resolution of 2 ns/address at 30 ° C. The traces shown were the average of 100 single laser flashes each. By analysis of the decay curves the following parameters were obtained: Trace (a) $r_0 = 0.1$, $\phi_1 = 1$ μs (41%), $\phi_2 = 105$ ns (59%); Trace (b) $r_0 = 0.12$, $\phi_1 = 205$ ns (22%), $\phi_2 = 49$ ns (78%). The lower part of the figure shows the corresponding residuals of the fit (upper trace two exponentials according to Eqn. 4, lower trace 1 exponential only). The standard deviation of the computer fit in the case of one exponential was about 3-times larger than the one of the two-exponential fit.

The observed decay of the absorption anisotropy may be attributed to one of the following motions: (a) rotation of the whole liposome containing the labelled protein; (b) rotation of the protein in the bilayer membrane; (c) segmental motion of polypeptide chains covering the label site with respect to the protein; (d) 'librational motion' of the dye with respect to the polypeptide chains [21–23,33,34,43].

These different kinds of randomization process, however, occur in different time domains [21,33,34]. We have previously shown that rotation of rather small liposomes with an average diameter of 30 nm prepared by the freeze/thaw technique in aqueous buffer solutions ($\eta \simeq 1$ mPa · s) occurred with a half-time of $\tau = 2$ μs [23]. With the applied dialysis technique, however, larger liposomes, with an average diameter of 300 nm, were obtained [39]. For spherical vesicles with $d = 300$ nm one would expect a rotational correlation time of about 3.5 ms (see Eqn. 6). Therefore, rotation of the vesicles can not account for the observed fast anisotropy decay (less than 1 μs) shown in Fig. 4b. On the other hand, librational motion of the protein-bound dye is very fast at room temperature (less than 10 ns [34]) and occurs at times which were not resolved in our measurements (see also Materials and Methods).

The mathematical analysis of the curve shown in Fig. 4b according to Eqn. 4 yielded the following parameters: initial anisotropy $r_0 = 0.17$; $a_1 = 0.045$; $a_2 = 0.12$; $\phi_1 = 200$ ns; $\phi_2 = 50$ ns. The theoretical limit of the initial absorption anisotropy for eosin, a linear absorber, is $r_0 = 0.4$ [33,34]. Due to experimental imperfections (scattering, depolarizations on optical surfaces) values of 0.38 can be measured [29,33,34,45]. Therefore, the apparently lower initial anisotropy in Fig. 4 can only be due to fast randomization processes, presumably to librational motion of the dye. According to the model of Saffman and Delbrück rotational diffusion of lipids and proteins in bilayer membranes may be considered as restricted to uniaxial rotation about the membrane normal [35]. In this case, the decay of the absorption anisotropy is described by the Eqn. 5 (Materials and Methods).

In order to attribute the observed anisotropy decay to one of the above depicted possible motions, we measured the temperature dependence of the absorption decay. The results of these measurements are shown in Fig. 5a, where the slower relaxation time, ϕ_1, as defined by Eqn. 4 is shown as a function of the temperature. The ratio of the experimentally obtained two rotational correlation times as function of the temperature is shown in Fig. 5b. As is obvious from this figure, the ratio ϕ_1/ϕ_2 between the two correlation times approaches a

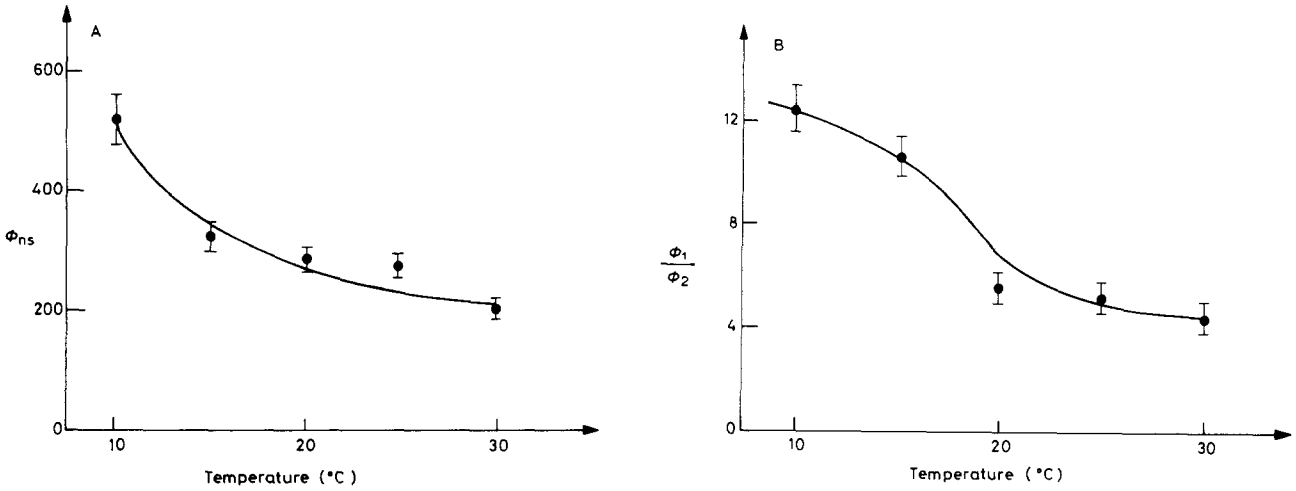

Fig. 5. Temperature dependence of the rotational correlation time for subunit-III-labelled CF_0CF_1 in azolectin liposomes. Rotational correlation time, ϕ_1, as defined in Eqn. 4 (A) and the ratio of ϕ_1/ϕ_2 at different temperatures (B). The rotational correlation times ϕ_1 and ϕ_2 were determined by the Provencher program according to Eqn. 4. Each point is the average of four independent experiments.

value of 4 at 30 °C. This factor strongly suggests that at temperatures of 30 °C and above, where the liposome membrane is in the liquid-crystalline state, the rotational diffusion of CF_0CF_1 labelled in subunit III in fact may be regarded as restricted and uniaxial rotation about the bilayer membrane normal. The rotational correlation time observed for uniaxial rotation of subunit-III-labelled CF_0CF_1 in the membrane was $\phi = 200 \pm 20$ ns at 30 °C. This rotational relaxation time for uniaxial anisotropic rotation of the labelled protein can be related to the size of the 'rotating unit' [33–35]:

$$\phi = (\mathbf{k} \cdot T)/(4 \cdot S_p \cdot h \cdot \eta_2) \quad (7)$$

where S_p is the cross-section area of the protein cylinder in the membrane, h is the thickness of the membrane and η_2 the 'effective viscosity' in the membrane, a quantity used to describe the dynamic friction in the membrane [35]. For bilayer membranes identical in composition to the one used in the present study, we have previously determined the friction in the membrane by measuring the rotation of lipid molecules [23]. We also have demonstrated that for high 'dilution' of the protein in the model membrane (lipid/protein > 10^4:1 mol/mol) at temperatures of 30 °C and above, where the membrane is in the liquid-crystalline state, rotational correlation times for protein and lipid rotation may be combined to obtain information on the protein cross-sectional area embedded in the lipid phase [23]. When the rotational diffusions of two different entities, which 'experience' the same effective friction in the membrane, are related to each other by Eqn. 5, one obtains [23]:

$$\phi_p/S_p = 2 \cdot \phi_1/S_1 \quad (8)$$

where ϕ_p and ϕ_1 are the rotational correlation times of the protein (ϕ_p) and the lipid (ϕ_1) and S_p, S_1 the corresponding cross-sectional areas of the two entities. With the rotational correlation time of 200 ns at 30 °C for subunit-III-labelled CF_0CF_1, the measured lipid rotation time of $\phi_1 = 20$ ns at 30 °C [23] and the known cross-sectional area of the lipid molecules $S_1 = 0.78$ nm^2 [47], one obtains $S_p \simeq 3.9$ nm or, since $S_p = \pi \cdot r^2$, a radius of 1.1 nm for the protein cylinder in the membrane. From this values we can exclude that we observed the rotational diffusion of the entire CF_0CF_1 complex in the liposome membrane.

The observed rotating unit in the membrane is probably composed of transmembrane α-helices. A single α-helix reveals a radius of $r \simeq 0.5$ nm; therefore the observed rotating unit should contain not more than two transmembrane helices. At most four transmembrane helices would be possible, but only if they are perfectly tightly packed. We have shown above that the EITC label was exclusively bound to subunit III (proteolipid) in the CF_0CF_1 complex. From secondary structure predictions a hairpin-like secondary structure, two transmembrane α-helices interconnected by a hydrophilic loop, has been postulated for the proteolipid [13]. This proposed structure fits well with the above calculated size of the observed rotating unit.

We therefore conclude that we have observed the rotational diffusion of the monomeric proteolipid. It appears that, after reconstitution into the liposome membrane, the EITC-labelled subunit III dissociates from the other subunits of the CF_0CF_1 complex.

Rotational diffusion of subunit-β-labelled CF_0CF_1 in the bilayer membrane

Fig. 4a shows the time-course of the absorption anisotropy of reconstituted subunit-β-labelled CF_0CF_1. The measurement shown was performed under the same

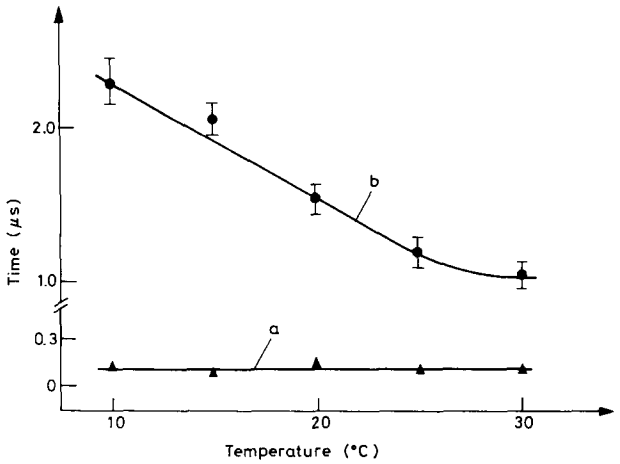

Fig. 6. Temperature dependence of the rotational correlation time for subunit-β-labelled CF_0CF_1 in azolectin liposomes. Trace (b) shows the correlation time ϕ_1 and trace (a) ϕ_2 at the indicated temperature. The rotational correlation times ϕ_1 and ϕ_2 were determined by the Provencher program according Eqn. 4. Each point is the average of four independent experiments.

experimental conditions as the measurements shown in trace 4b. Obviously, the anisotropy in trace a decays more slowly than in trace b. The computer fit of trace 4a according to Eqn. 4 revealed two decay components with correlation times of 1 μs and 80 ns, respectively. In order to attribute the observed anisotropy decay for subunit-β-labelled CF_0CF_1 to one of the possible motions (see above), we also measured the temperature dependence of the absorption anisotropy decay in the proteoliposomes containing subunit-β-labelled CF_0CF_1. At all temperatures the decay curves could be fitted by Eqn. 4. In Fig. 6 the two relaxation times obtained are plotted as a function of the temperature. This figure shows that the faster relaxation time remained constant over the temperature range shown (80 ± 18 ns). However, the slower relaxation time revealed a marked temperature dependence, increasing with decreasing temperature. These findings are quite different from the one obtained above for subunit-III-labelled CF_0CF_1. However, in rotational diffusion measurements in solution with isolated CF_1, which has also been labelled in the β subunit with EITC, we previously also obtained an anisotropy decay component which was rather insensitive to the viscosity of the buffer [43–45]. We therefore had to conclude that this decay component was due to restricted mobility of the label in respect to the β subunit of CF_1. In the present study, similar to these results, the triplet probe was bound to the β subunit and the anisotropy decay revealed a component with a time constant of about 80 ns independent of the medium viscosity. We therefore tentatively conclude that only the slower decay component in Fig. 6 can be attributed to the rotational diffusion of CF_0CF_1 in the liposome membrane. With the temperature dependence given, the decay time of this slower component approaches an almost temperature-independent value of $\phi_1 \approx 1$ μs at temperatures above 25 °C. Similar behaviour was typically observed for uniaxial protein rotation in membranes [21–23]. For uniaxial protein rotation one would expect two decay components according to Eqn. 5 in addition to the above 80 ns decay component. However, at present the three closely spaced decay components are far beyond experimental resolution. We therefore interpret the slower relaxation time in Fig. 5 as apparent rotational correlation time ϕ_{app}, which in combination with the time-independent component, a_3 (see Eqn. 5), can be used to calculate the rotational correlation time, ϕ [34]:

$$\phi_{app} = (1/D_{\parallel}) \cdot (1 + 15 \cdot \cos^2\theta)/(4 + 12 \cdot \cos^2\theta) \quad (9)$$

where θ is calculated from:

$$a_3 = 1/10 \cdot (3 \cdot \cos^2\theta - 1)^2 \quad (10)$$

The time-independent residual anisotropy was $a_3 = 0.005$ (normalized to an initial anisotropy of $r_0 = 0.4$). From this value, two possible values for θ, $\theta = 50°$ and $\theta = 60°$ can be calculated [34]. With $\phi_{app} = 1$ μs one obtains a rotational correlation time of $\phi = 1.3$ μs ($\theta = 50°$) or $\phi = 1.5$ μs ($\theta = 60°$) for the rotational diffusion of the subunit-β-labelled CF_0CF_1 in the liposome membrane. Applying the above described formalism with Eqn. 8 we then obtain a cross-sectional area of 26–29 mm^2 and a diameter of 5.8–6.1 nm for the 'rotating unit'. We tentatively assign this rotational correlation time of $\phi = 1.3$–1.5 μs to the rotational diffusion of the entire CF_0CF_1 complex in the liposome membrane. The rotational diffusion of the complex would then be determined mainly by the friction experienced by CF_0 within the membrane.

A main difficulty in the interpretation of protein rotational diffusion in membranes arises from the lack of knowledge of the dynamic friction in the membrane [22,23,33,49]. We previously measured the rotational diffusion of EITC-labelled $Pam_2GroPEtn$ in the membrane of azolectin liposomes and obtained a rotational correlation of $\phi = 20 \pm 5$ ns at 30 °C for the restricted rotation of the lipids about the membrane normal [23]. The results presented here clearly indicate that, according to the model of Saffman and Delbrück [35], we observed mainly restricted rigid body rotation about the membrane normal with the reconstituted subunit-III-labelled enzyme as well as with the subunit-β-labelled enzyme. We may therefore to a good approximation use the above rotation time of the lipids to convert the rotational correlation times of subunit III and subunit-β-labelled CF_0CF_1 in the azolectin membrane into the size of the 'rotating unit' (see Eqn. 8). For subunit-III-labelled CF_0CF_1 we obtained a 'protein cylinder' in the membrane with a cross-sectional area of $S_p \simeq 3.9$ nm^2

and a corresponding radius of about 1.1 nm. The 'rotating unit' probably consists of membrane-spanning connected α-helices as observed for other membrane proteins [51]. Considering known features of membrane proteins [51], however, the size of the rotating unit calculated above would allow only two transmembrane-connected α helices. This is the size of the monomeric proteolipid proposed from CD spectra [50] and secondary structure predictions [13]. From the above we feel confident that we in fact observed rotation of the monomeric proteolipid. In CF_0CF_1 the proteolipid oligomer appeared to act in cooperative manner thus forming a functional unit, which specifically conducts protons across the membrane [1,2,9,13]. Generally, the oligomeric form of the proteolipid was thought to be very stable [1,2,9,13,37]. Even in SDS-PAGE gels the proteolipid was found in higher oligomeric forms which hardly could be disrupted [11,52,53]. Our results, however, indicate that the proteolipid oligomer in artificial liposome bilayer membranes easily dissociates into the momomeric form after modification with EITC. Another line of evidence suggests that this dissociation of the EITC-labelled proteolipid may be reversible, at least when a membrane potential is applied across the liposome membrane. We fused proteoliposomes containing subunit-III-labelled CF_0CF_1 to giant liposomes according to Criado and Keller [54]. By employing patch clamp techniques, we observed cation-conducting channels with at least three different conductance stages (about 15, 30, 60 pS, symmetrical 10 mM Tris-HCl (pH 6.8)/120 mM NaCl in the membrane of this giant liposomes (Wagner, R. and Lühring, H., unpublished data). Since it seems very unlikely that the monomeric EITC-labelled proteolipid can form cation channels [11], it is probable that the labelled proteolipid reassociates to form cation-conducting oligomers, at least during the time in which the membrane potential is switched on.

For subunit-β-labelled CF_0CF_1, about 28 nm^2 was calculated for the cross-sectional area of the rotating unit. The validity of this calculation requires that the dimensionless parameter ϵ, which characterizes the model of Saffman and Delbrück [35] is smaller than 1 [55]:

$$\epsilon = (\eta_1' + \eta_1'') \cdot a / \eta_2 \cdot h \cdot V \qquad (11)$$

where η_1' and η_1'' are the bulk viscosities on both sides of the membrane, a is the radius of the rotating cylinder in the membrane and h the thickness of the membrane. The CF_1 part protrudes about 12 nm from the membrane [41]. This will significantly increase the drag forces on the entire enzyme complex when $\epsilon > 1$ [55]. With $\eta_1' = \eta_1'' \approx 1$ mPa · s, $\eta_2 \approx 12$ mPa · s [23], $h \approx 5$ nm and $a \approx 3$ nm we obtain $\epsilon \approx 0.1$. This value is low enough to justify the application of the Saffman and Delbrück equations [35,55] and therefore the CF_0 part may be in a fair approximation considered as the rotating unit in the membrane. Moreover, the calculated value of about 6 nm for the diameter of CF_0 is in good agreement with the electron microscopy data of Boekema et al. [41] for CF_0, which additionally supports our above conclusion.

Together, our measurements of the lipid rotational diffusion [23] and rotational diffusion of membrane proteins of different sizes, chloroplast-triose-phosphate-3-phosphoglycerate translocator (29–34 kDa [23,56]), subunit III (8 kDa) of the chloroplast ATP synthase and the whole CF_0 part of the enzyme (about 150 kDa) provide a quite coherent picture. They demonstrate, in agreement with previous reports [22,23,49], that the model of Saffman and Delbrück can be used to describe the diffusion of lipids and proteins in membranes, provided the membrane is in the liquid-crystalline state and the proteins are highly diluted in the membrane. The membrane viscosity as probed by the lipid molecules and proteins of different size appeared to be the same and was for the azolectin membrane in the order of 12 mPa · s [23].

We used the results of the rotational diffusion measurements on CF_0CF_1 and CF_1 to calculate some overall dimensions of the CF_0CF_1 complex compatible with the measured rotation times. The results of this calculation are depicted in Fig. 7, which shows an arbitrary model structure with our calculated values. It is worth mentioning that our calculated values are in good agreement

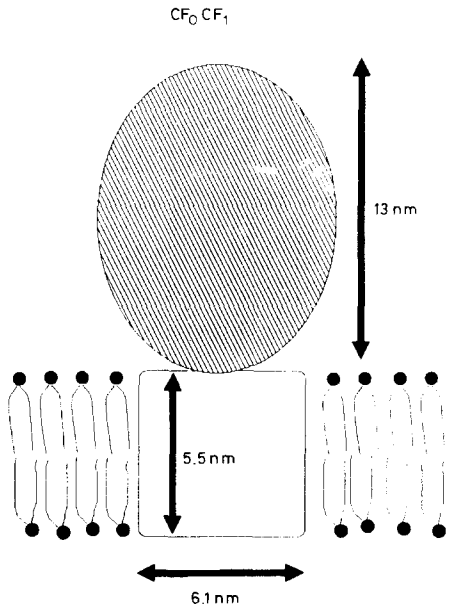

Fig. 7. Model of the CF_0CF_1 complex with the distances calculated from the rotational correlation times. The dimensions were calculated from the three measured rotational correlation times (see Results) of CF_1 in solution (320 ns; $d = 13$ nm), the CF_0CF_1 micelle in solution (820 ns; $d = 18.5$ nm) and CF_0CF_1 reconstituted in the membrane (1.3–1.5 μs in liposomes). (Ref. 40, for details see text.)

TABLE I

Effect of venturicidin and tributyltin on the rotation of subunit-III- and subunit-b-labelled CF_0CF_1 in liposomes.
For details see text

Sample	Rotational correlation time (ϕ)
CF_0CF_1, subunit III labelled	200 ns
CF_0CF_1, subunit III labelled + venturicidin/tributyltin	200 ns
CF_0CF_1, subunit β labelled	1.3–1.5 μs
CF_0CF_1, subunit β labelled + venturicidin/tributyltin	1.9–2.1 μs

with the overall dimensions of CF_0CF_1 as obtained by electron microscopy [41].

Effect of venturicidin and tributyltin on the rotational diffusion of reconstituted CF_0CF_1

We further investigated the effect of the inhibitors of proton flow through CF_0, venturicidin and tributyltin [48] on the rotational diffusion of reconstituted subunit-III- and subunit-β-labelled CF_0CF_1. Incubation of proteoliposomes containing subunit-III-labelled CF_0CF_1 with both energy transfer inhibitors did not change the rotational correlation times within the limits of error .

However, both inhibitors increased the rotational correlation times of reconstituted subunit-β-labelled CF_0CF_1. This is shown in Table I, where the rotational correlation times, ϕ, as calculated from the measured apparent rotational correlation times (ϕ_{app}, see above) are listed. Since neither inhibitor changed the rotation times of the monomeric EITC-labelled subunit III, the observed increase from $\phi = 1.3–1.5$ μs to $\phi = 1.9–2.1$ μs indicates that binding of the inhibitors changes the overall structure of the CF_0 part drastically.

The increased rotation time has to be attributed to an apparent increase of the cross-sectional area of CF_0. Binding of the small inhibitor molecules to CF_0 alone can hardly account for the observed increase in the rotational correlation times. Therefore we have to conclude that both inhibitors change the overall structure of CF_0 required for specific proton transport across the membrane.

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