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Rotational mobility and domain flexibility of membrane-bound bacterial coupling factor as detected with the triplet probe eosin-isothiocyanate

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The membrane-bound coupling factor (BF_1) of chromatophores from the photosynthetic bacerium *Rhodopseudomonas sphaeroides* was covalently labeled with the triplet probe eosin-isothiocyanate. The labeled enzyme was isolated and functionally reconstituted into depleted chromatophores from the same bacterium. ATPase and ATP synthase activities of the reconstituted vesicles were strongly dependent on the labeling conditions, decreasing at increasing load of eosin molecules per BF_1. When labeling was carried out in the dark and in the presence of ATP, one molecule of eosin isothiocyanate was bound per BF_1 and the activities catalyzed by the reconstituted and labeled enzyme were as high as in untreated chromatophores. Upon light energization of the chromatophore membrane, a large conformational change of BF_1 could be detected by using the triplet probe as a spectroscopic tool. The domain flexibility and rotational mobility of the reconstituted coupling enzyme were directly related to the enhancement of the ATPase activity induced by light. Both the light-stimulated ATPase activity and conformational changes could be prevented by addition of ADP or oligomycin and affected to the same extent by uncouplers and inhibitors of electron transport. Moreover, the detected conformational changes were reversible in time, appearing with a half-time of 10 ms upon illumination of the chromatophores, and disappearing with a half-time of 70 ms in the dark. The results obtained prove the feasibility of the spectroscopic technique in detecting conformational changes of the membrane-bound BF_1, similarly to what already has been observed for chloroplast coupling factor (Wagner, R. and Junge, W. (1980) FEBS Lett. 114, 327–333), and add to the possibility of characterizing, by this method, energy transduction at a molecular level.

Introduction

Photosynthetic ATP synthesis in green plants and bacteria is catalyzed by membrane-bound oligomeric enzyme complexes, designated CF_0-CF_1 and BF_0-BF_1, respectively. These enzyme complexes consist of two separable moieties, a hydrophobic intrinsic membrane part (CF_0 and BF_0) and a water-soluble peripheral part (CF_1 and BF_1). The membrane sector is involved in proton translocation across the energy conserving membrane, while the water soluble headpiece contains the catalytic site (for a review, see Refs. 1 and 2).

As proposed by Mitchell almost two decades ago, many published results are indicating that the ATPase complex acts as a reversible, ATP-depen-

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Abbreviations: BChl, bacteriochlorophyll; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential difference of protons; P_i, disodium hydrogen phosphate.

dent proton pump. A similar number of subunits was found for the two moieties, rather independently of the source: generally five polypeptides assemble to constitute the water-soluble part and three or five can be isolated from the membrane sector (for a review, see Refs. 3–6). In comparison with other ion pumps, the structure and assembly of the ATPase is therefore rather complex and it still remains elusive, although the aminoacid sequence of the individual subunits became in part available [2]. In spite of a large number of studies, the molecular mechanism of ATP synthesis remains, however, to be characterized especially in relation to the possible mode of energy transduction.

Conformational changes of the enzyme complexes seem to play an essential role during coupling processes [6,7]. They were first inferred from chemical modification studies, characterization of nucleotide binding, and detection of fluorescence changes of covalently bound fluorophores [6,8]. It is not known, however, whether they reflect regulatory events and/or energy-transducing steps during the catalytic activity. More informations on the functional mechanism at a molecular level can be obtained when methods, useful to detect, characterize and time-resolve segmental motions of membrane proteins, are utilized. A spectroscopic technique, based on the use of triplet probes as spectroscopic tools covalently attached to the active enzymes, and introduced by Cherry et al. [9], was developed and utilized to detect conformational changes of isolated and membrane-bound coupling factors [10] as well as of other membrane proteins such as ferrodoxin-NADP-oxidoreductase [11] and the Ca^{2+}-Mg^{2+} dependent ATPase of the sarcoplasmic reticulum [12]. In these studies, the proteins under investigation are covalently labeled with the triplet probe eosin isothiocyanate which, upon excitation with a short laser flash, can be transformed into a relatively long lived triplet state. Conformational changes of the enzyme are followed by spectroscopically detecting both the triplet probe lifetime and its absorption anisotropy under photoselection [13]. The triplet lifetime of the bound molecule depends on the access of the most potent triplet quencher oxygen to a given binding site in the host protein. It is therefore very sensitive to the proximity of a binding site to the bulk medium and/or to the flexibility of the protein chains which cover it [11]. On these bases, it is possible to discriminate binding sites at the protein surface or within a very flexible protein, since they are better accessible to bulk oxygen than those inside a rigid protein matrix. Moreover, the decay of the absorption anisotropy of the triplet probe allows to follow the mobility of the dye relative to the protein ('librational motion') and the rotational diffusion of the whole protein in the membrane.

In the absence of a light-dependent $\Delta\tilde{\mu}_{H^+}$, membrane-bound CF_1 behaves as an apparent irreversible ATP synthase [6]. Bacterial chromatophores, on the contrary, show a manifest ATPase activity, which, in addition, in the absence of ADP, can be activated by light [14]. It can be, therefore, useful to monitor the conformational changes of membrane bound BF_1 in order to test further the reliability of this method in time-resolving the domain flexibility of coupling enzymes during the catalytic activity, induced by light-dependent energization of the photosynthetic membrane. A comparison of the results obtained with coupling enzymes, which act similarly, although different in a main aspect, will better characterize the role of conformational changes in energy transduction.

In this paper, we report on the labeling of membrane bound BF_1 of chromatophores from *Rhodopseudomonas sphaeroides* with eosin isothiocyanate. Furthermore, we characterize the influence of the bound label on the activity of the reconstituted enzyme, measure the rotational diffusion of the membrane-bound labeled enzyme and time-resolve its conformational changes during the light-dependent activation of the ATPase activity and its modulation by uncouplers, inhibitors and substrates of photophosphorylation.

Materials and Methods

Biochemical procedures

Chromatophores were prepared from cells of *Rhodopseudomonas sphaeroides*, strain Ga, grown photosynthetically and harvested routinely at the end of the logarithmic phase, as described previously [15]. Bacteriochlorophyll (BChl) content was measured in aceton/methanol extracts following

the procedure reported in Ref. 16. Protein concentration was determined by the Coomassie brilliant blue method [17]. Eosin isothiocyanate was prepared by bromination of fluorescein isothiocyanate (Sigma, isomer I), following the procedure of Cherry et al. [9]. The concentration of the dye was determined spectroscopically by using an extinction coefficient of $8.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for the peak at 530 nm.

Labeling of membrane-bound BF_1 was performed as essentially outlined in Ref. 10, with the following modifications. Chromatophores were suspended in an assay medium containing 50 mM tricine (pH 8.3), 5 mM $MgCl_2$, 10 mM NaCl and 0.2 mM sodium succinate at a final BChl concentration of 25 μM. Eosin isothiocyanate, which was dissolved immediately before use in 0.1 mM phosphate buffer (pH 8.3) at a concentration of 4 mg/ml, was added to the chromatophore containing assay medium at a BChl/eosin weight ratio of 3.5. The reaction was carried out for 5 min, either under illumination with red light (0.7 W/cm^2) or in the dark. The final concentration of ATP, when present, was 4 mM. The samples were contained in glass test tubes, continuously stirred and thermostated at 25°C. The incubation period was terminated by adding 100 mM glycine in order to trap the unreacted eosin isothiocyanate. The suspension was then 10-fold diluted with a 50 mM glycylglycine buffer (pH 7.5), containing 5 mM $MgCl_2$, and centrifuged at $200\,000 \times g$ for 30 min. Most of the unbound eosin remained in the supernatant, which was discharged. To remove unspecific adsorbed eosin, the pellet, resuspended in the same dilution buffer at a concentration of approx. 4 mM BChl, was passed through a Sepharose 6B column (100×5 cm) and eluted with the void volume.

Labeled or unlabeled BF_1 was resolved from chromatophores (suspended in a medium containing 4 mM EDTA at a concentration of about 2–3 mM BChl) by sonication in the cold for 90 s (three bursts of 30 s) with a Branson sonifier B15, equipped with a microtip vibrating at maximal amplitude. The crude supernatant, obtained by centrifugating the suspension for 30 min at $200\,000 \times g$ was concentrated by ultrafiltration with a Diaflo YM-10 membrane, to approx. 500 μg protein/ml. When containing labeled BF_1, the concentrated sample was passed through a small Sephadex G-25 column equilibrated with the standard glycylglycine buffer according to the protocol of Penefski [18]. The latter step was performed in order to remove eosin not covalently bound to proteins. The eosin eluted from the column with the crude supernatant, still resided on the proteins after heating the sample with SDS (1%) at 60°C for 10 min, following the procedure described in Ref. 19, and was therefore at least 95% covalently bound. BF_1, when necessary, was purified at 4°C on a Sephadex G-200 column (80×2.6 cm), as described in Ref. 19.

Gel electrophoresis was applied as described in Ref. 20, either in the presence or absence of SDS. To quantitate the amount of eosin per BF_1, bands as detected by ultraviolet spectroscopy, were sliced, homogenized and protein extracted from the gels following the procedure described in Ref. 21. The samples were then filtered through 0.45 μm millipore filters and the phosphorescence of eosin was recordered at 555 nm (excitation at 520 nm) in an SLM spectrofluorimeter. Eosin concentration was determined by calibration of the phosphorescence with samples of known concentration. The protein concentration of the single bands was determined as in Ref. 17. BF_1 concentration was calculated by using a molecular weight of 380 000, as determined from gel chromatography on Sephadex G-200. The following proteins were used as markers: aldolase, bovine serum albumin, catalase and ferritin.

Reconstitution of BF_1 was performed by incubation of depleted chromatophores with the crude supernatant at 25°C for 20 min (in the presence of 50 mM KCl and 10 mM $MgCl_2$). Approx. 100–200 μg protein were added to a 2 ml suspension containing depleted chromatophores corresponding to 20–30 μg BChl. After incubation, the supernatant was isolated from the reconstituted chromatophores by passing through a Sepharose 6B column, as described above.

ATPase and ATP synthase activities of membrane-bound BF_1 were determined according to standard procedures [21]. The rate of ATP synthesis or hydrolysis was determined from the incorporation or release of $^{32}P_i$ into or from $^{32}P[ATP]$, respectively. The sample, in a 1 ml final volume, contained 50 mM tricine (pH 8)/50 mM KCl/5 mM $MgCl_2$/0.2 mM succinate/20–40 μM BChl.

ADP and P_i, when necessary, were added at a 4 mM final concentration. Light activation of membrane bound BF_1 was carried out by preilluminating chromatophores for 2 min, in the presence or absence of the suited nucleotides, as described in Ref. 23. The weight ratio of oligomycin, when present, to BChl was 0.3. Buffer viscosity was measured with a Uppelohde viscosimeter.

Flash spectrophotometry

Flash spectrophotometric experiments and photoselection studies were performed as outlined in Ref. 19. In photoselection studies, the monitoring beam (from a tungsten iodine lamp) was linearly polarized (by a rotatable Glan-Thomson polarizer) either parallel or perpendicular to the exciting light. Non-saturating flash energies for excitation were provided by an intrinsically polarized frequency-doubled Q-switched Nd-YAG laser with a maximal output of 20 mJ per flash. By using neutral density filters in front of the laser beam, less than 30% of the eosin molecules were excited. The assay medium was the same as that previously described for photophosphorylation. The samples (1 ml), containing chromatophores (40 μM BChl) reconstituted with eosin-labeled BF_1 in 60% (v/v) glycerol, were deoxygenated by a stream of argon (for 10 min) and passed to a 1 cm optical cell with a thermostated glass mantle sealed against oxygen. Glycerol and deoxygenation were routinely omitted when the samples were only assayed for the triplet probe lifetime.

Flash-induced absorption changes of eosin at 542 nm, indicating ground state depletion, were recorded with a Biomation transient recorder and averaged with a digitally interfaced Tracor averaging computer (30 and 100 traces were averaged when the triplet lifetime and the linear dichroism were measured, respectively). At this wavelength, contribution of the intrinsic electrochromic absorption changes of the chromatophores (mainly due to endogenous carotenoids) were at least 10-fold smaller than the eosin absorption changes. Moreover, they were constant in the time scale of the experiments and therefore neglected.

The absorption anisotropy parameter $r(t)$ was calculated from the equation: $r(t) = A_{\parallel}(t) - A_{\perp}(t)/A_{\parallel}(t) + 2A_{\perp}(t)$, where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorption changes for parallel and perpendicular polarization between the measuring and exciting light. Analysis of the data was performed with a PDP11/34 and a TR440 computer by means of a Fourier method for the analysis of curves with an exponential decay [24].

White or red side illumination was provided by a halogen lamp switched (on and off) by a photoshutter. Light intensity was 0.7 W/cm^2 and illumination was carried out as specified. If not otherwise specified, the laser flash was usually fired either 5 or 10 ms after switching off the side illumination.

To detect possible eosin-sensitized photoinactivation, the ATPase activities of the samples were measured before and after flash photolysis: no significant variation was noticed.

Results

Labeling of BF_1 with eosin isothiocyanate and reconstitution of depleted chromatophores with labeled BF_1

Eosin isothiocyanate reacts preferentially with ϵ-aminogroups of lysin or terminal aminogroups of proteins [25]. The exposure of chromatophores to the dye caused, therefore, an unspecific labeling of all accessible membrane proteins. For this reason, special precautions were necessary to obtain a chromatophore preparation selectively labeled on BF_1. The enzyme, contained in the crude supernatant of a sonicated suspension of chromatophores from *Rps. sphaeroides*, could be purified to homogeneity by gel filtration. The purified BF_1 had an apparent molecular weight of approx. 380000 and showed five bands on SDS-polyacrylamide gel electrophoresis (results not shown; see also Ref. 26). However, on the contrary of purified CF_1 and similarly to other preparations of purified BF_1 [14], the enzyme lost its ATPase activity and its capability to rebind to depleted chromatophores, usually within two days, also when other extraction procedures were used [14]. Therefore, it could not be successfully used to prepare a chromatophore population with a selectively labeled, but still active coupling enzyme. In this respect, we found it suitable and successful to follow a three-step procedure: (1) labeling of chromatophores with eosin; (2) detachment of labeled membrane proteins (including BF_1) by brief soni-

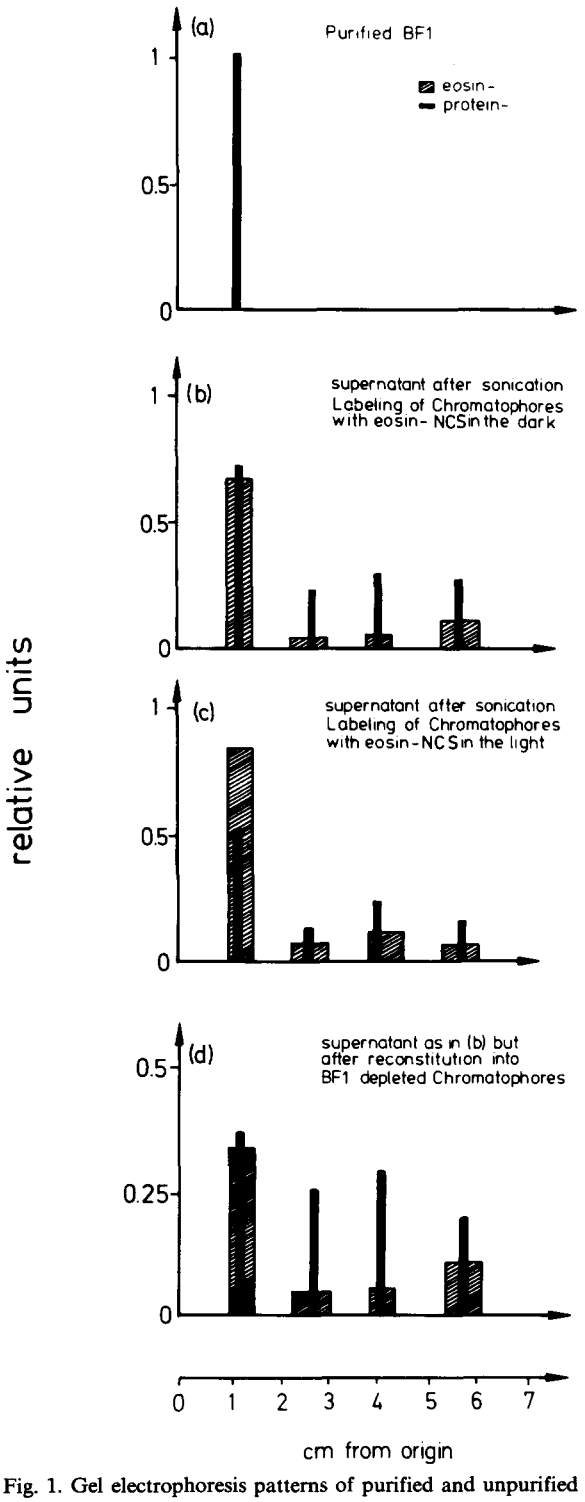

Fig. 1. Gel electrophoresis patterns of purified and unpurified labeled BF_1. Preparative gel electrophoresis of purified unlabeled BF_1 (a), and of a BF_1-containing supernatant obtained after sonication of chromatophores exposed to eosin isothiocyanate in the light (b) and in the dark (c), respectively; (d), the same as in (b) after reconstitution by BF_1-depleted chromatophores. The eosin content of the single bands is also shown. Procedures are detailed under Materials and Methods. Eosin and protein concentrations are expressed as relative units of 0.91 nM and 100 μg respectively.

cation in the presence of EDTA; (3) reconstitution of depleted, unlabeled membranes with the partial purified extract from step 2. The supernatant from step 2 could also be used for the separation of the different proteins with polyacrylamide gel electrophoresis and determination of bound eosin in the BF_1 fraction.

Chromatophores from *Rps. sphaeroides* were labeled with eosin isothiocyanate either in the dark or in the light, and sonicated in order to extract the labeled BF_1. The crude supernatant, obtained after centrifugation of the sonicated suspension, was tested for its BF_1 content with polyacrylamide gel electrophoresis and the protein and eosin concentrations of the single bands were determined. The results of this analysis are shown in Fig. 1. As a control, the relative mobility of purified unlabeled BF_1 is also shown (Fig. 1a). It is evident that the crude supernatant contained, beside BF_1, at least three other detectable and not identified proteins (Fig. 1b). The relative amount of BF_1 to the total protein content was about 55% and the load of eosin per protein (especially per BF_1) was higher when labeling was carried out in the light than in the dark (compare c with b in Fig. 1). After incubation with depleted chromatophores, the crude supernatant was separated from the reconstituted chromatophores and again analyzed with gel electrophoresis (as an example, compare d with c in Fig. 1). Only the protein content of the band corresponding to BF_1 was lowered (to about 30% of the total protein content), indicating that, of the four proteins released from chromatophores after sonication, only BF_1 was significantly rebound to the depleted membranes.

Chromatophores were, therefore, reconstituted with the supernatant containing eosin-labeled BF_1; the ATPase and ATP synthase activities, catalyzed by these preparations, were measured and compared to those of untreated chromatophores. The results of a typical set of experiments, performed utilizing different preparations of chromatophores,

TABLE I

RECONSTITUTION OF DEPLETED CHROMATOPHORES WITH UNLABELED BF_1: ATPase AND ATP SYNTHASE ACTIVITIES

Activities are expressed as micromoles ATP hydrolyzed or synthesized per h per mg BChl. The figures are the mean values of three independent measurements; accuracy for each determination was within 10%.

	Additions	ATPase		ATP synthase
		dark	light	
Control	none	27	49	170
	+ oligomycin	13	2	1
After depletion	none	10	17	40
	+ oligomycin	7	2	1
After reconstitution	none	46	67	90
	+ oligomycin	5	1	1

are shown in Tables I, II and III. Chromatophores of *Rps. sphaeroides* are endowed with both an ATPase and an ATP synthase activity, the values of which may change mainly depending on the growing conditions of the cells [15]. As a general feature, the ATPase activity, which is manifest in dark adapted membranes, can be either Ca^{2+}- or Mg^{2+}-dependent and is about 50% inhibited by oligomycin [14]. Only the Mg^{2+}-dependent ATPase activity can be, however, light activated under conditions optimal for cyclic electron flow (in the presence of 0.2 mM succinate [14]). The rate of

TABLE III

MODULATION OF ATPase AND ATP SYNTHASE ACTIVITIES IN CHROMATOPHORES RECONSTITUTED WITH EOSIN-LABELED BF_1

Labeling was performed in the dark in the presence of ATP (0.9 eosin/BF_1). Oligomycin-sensitive activities are expressed as in Table I. The dark ATPase, light ATPase and ATP synthase activities of control chromatophores in this preparation were 30, 60 and 200 μmol ATP hydrolyzed or synthesized per h per mg BChl, respectively. Antimycin, valinomycin and nigericin concentrations were 0.2, 2 and 2 μM, respectively.

Additions	ATPase		ATP synthase
	dark	light	
None	27	57	150
ADP	28	28	
P_i	31	54	
ADP and P_i	26	46	
Antimycin	25	37	22
Valinomycin	33	46	78
Nigericin	41	66	150
Valinomycin and nigericin	20	20	2

ATP hydrolysis, measured in the light upon energization of the chromatophore membrane, is 2-fold higher than that in the dark, and it is almost totally impaired by oligomycin. After detachment of BF_1 by sonication, the Mg^{2+}-dependent ATPase and ATPsynthase activities are severely decreased and can be almost totally restored by incubating the BF_1 containing crude supernatant with the

TABLE II

RECONSTITUTION OF DEPLETED CHROMATOPHORES WITH EOSIN-LABELED BF_1: ATP-ase AND ATP SYNTHASE ACTIVITIES

Activities are expressed as in Table I. Molar ratios of eosin/BF_1 were evaluated as detailed in Materials and Methods. Control chromatophores were endowed with dark ATPase, light ATPase and ATP synthase activities of 40, 80 and 400 μmol ATP hydrolyzed or synthesized per h per mg BChl, respectively. This preparation was obtained from cells harvested at the early logarithmic phase of growth.

Labeling conditions	Eosin/BF_1	Additions	ATPase		ATP synthase
			dark	light	
In the light, with ATP	2.2	none	25	49	170
		+ oligomycin	7	9	1
In the light, without ATP	3.0	none	12	10	30
		+ oligomycin	5	1	1
In the dark, with ATP	0.9	none	39	80	350
		+ oligomycin	14	6	2
In the dark, without ATP	1.7	none	21	10	40
		+ oligomycin	10	1	1

depleted membranes (Table I). The Mg^{2+}-dependent dark and light ATPase and ATP synthase activities of depleted chromatophores, reconstituted with labeled BF_1, depend on the different conditions used for labeling (Table II). The number of eosin molecules covalently bound to BF_1 and determined from experiments similar to those shown in Fig. 1, decreases in the presence of ATP, if labeling occurs either in the light or in the dark. From our results (data not shown), the label is to be located most probably in the α and β subunits of BF_1, as experienced with coupling factors from other sources [27].

The presence of ATP during incubation with eosin preserve the capability of the reconstituted system to be activated by light. It is noticeable that the most active vesicle preparation, endowed with activities comparable to those of control chromatophores, is obtained by reconstituting with BF_1 labeled in the dark and in the presence of ATP, at a load of approx. one molecule of eosin per BF_1.

Laser flash spectrophotometry of chromatophores reconstituted with eosin-labeled BF_1

Triplet lifetime of eosin bound to BF_1. The time-course of eosin absorption changes at 542 nm, after flash excitation of a suspension of depleted chromatophores reconstituted with eosin-labeled BF_1 is shown in Fig. 2. The absorption changes reflect the ground state depletion of eosin molecules and its subsequent repopulation from the triplet state. Depleted chromatophores reconstituted with BF_1 labeled in the light (Fig. 2A and C) and in the dark (Fig. 2B and D) in the presence of ATP, were utilized. The traces shown in Fig. 2A and B were recorded when the chromatophores were dark adapted, whereas those of Fig. 2C and D were performed after providing actinic light for 1 s before the laser flash. The results indicate that the decay of the eosin absorption changes is very different depending on whether BF_1 was labeled in the light or in the dark. When labelling of BF_1 had occurred in the presence of ATP and in the light, the time necessary to repopulate the ground state of eosin molecules covalently bound to the reconstituted BF_1, was longer than that necessary when labeling had been carried out in the dark (compare Fig. 2A to B). The evaluated lifetimes were 230 and 32 μs for the light- and dark-labeled samples,

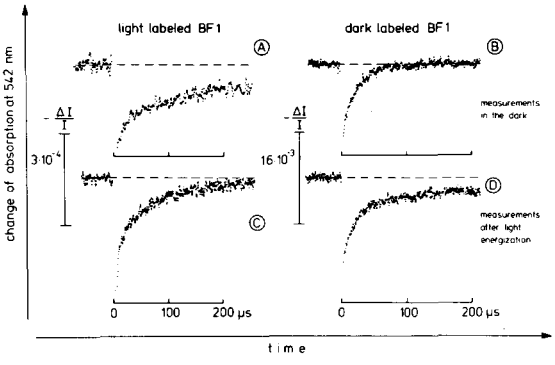

Fig. 2. Absorption changes of eosin at 542 nm in chromatophores reconstituted with eosin-labeled BF_1. Measurements in A and B were performed in the dark, whereas those in C and D were recorded after energization of the chromatophore membrane with actinic light for 1 s. A and C, chromatophores reconstituted with BF_1, labeled in the light in the presence of ATP (2.2 mol eosin/BF_1); B and D, chromatophores reconstituted with BF_1, labeled in the dark in the presence of ATP (0.9 mol eosin/BF_1).

respectively. Since the triplet lifetime is strongly dependent on the accessibility of oxygen to the excited molecules, these data indicate that the eosin binding sites are closer to the protein surface in the latter than in the first case. When side illumination was provided to the samples, the triplet lifetime of the eosin chromophores shortened (Fig. 2C) or elongated (Fig. 2D), again depending on whether labeling had occurred in the light or in the dark. Moreover, in both cases, it decayed biphasically with half-times of 38 and 150 μs for the light-labeled, and 23 and 220 μs for the dark-labeled samples. Addition of oligomycin to the samples prevented the change of the triplet lifetime due to energization of the chromatophore membrane in both preparations (data not shown). When the lifetime of the triplet was measured in samples containing chromatophores reconstituted with BF_1-labeled either in the dark or in the light, but in the absence of ATP, no difference from the time course shown in Fig. 2A and 2B was detectable (data not shown). However, and most noticeably, the side illumination effect was totally absent, indicating a close parallelism between the observed change in triplet lifetime and the increase in the light-activated ATPase activity of the reconstituted chromatophore population. Furthermore, the membrane energization-dependent change on

the triplet lifetime was also modulated by known effectors of the ATPase and ATP synthase activities. ADP added at concentrations which prevent the stimulation of the ATPase activity in the light (as is shown in Table III) prevents also the side-illumination-induced change of the lifetime of the bound triplet probe. When P_i was added to the ADP-containing sample, the effect of ADP addition was completely reversed, as is shown in Fig. 3. This effect was again sensitive to oligomycin. Finally, it is worth to note that valinomycin and nigericin abolished the observed effects only when added together at a concentration of 2 μM, which in this preparation abolished both the light-dependent ATPase and ATP synthase activities (Table III) and it is known to impair the light-induced $\Delta\tilde{\mu}_{H^+}$ in untreated chromatophores totally [28]. Antimycin was effective at a concentration (0.2 μM) which abolish both the rate of photophosphorylation and the light stimulation of the ATPase activity, without affecting the total $\Delta\tilde{\mu}_{H^+}$ (traces not shown, Table III and Ref. 28). All together, these results clearly demonstrate that the observed changes in the triplet lifetime of the eosin probe covalently bound to the reconstituted BF_1 are directly related to the enzyme activity modulated by the energization of the chromatophore membrane. Moreover, the light induced changes in the triplet lifetime were also reversible in time. This was measured by detecting the time course of the absorption change of the BF_1 bound triplet probe as a function of the dark time after membrane energization, in chromatophores reconstituted with BF_1 labeled in the dark and in the presence of ATP. It was shown above that the main feature of the light-induced change of the eosin absorption in a chromatophore population reconstituted with labeled BF_1, was that the signal from monophasic became biphasic (compare Fig. 2B and 2D). The half-time of the two components detected upon energization of the chromatophores were about the same, independently of the illumination time (when side illumination was provided for 10 ms instead of 1 s, the half-times were 20 and 120 μs respectively (trace not shown) instead of 23 and 220 μs as in Fig. 2D); the relative amplitude of the two components was, however, dependent on the illumination time before laser flash. This flash, fired at the end of a 10 ms illumination period, was considered as $t = 0$. Additional measurements with the laser flash fired from 5 up to 200 ms after termination of the side illumination, allowed then to detect the time-course of the light induced change of the triplet lifetime. The results are shown in Fig. 4, where the relative amplitudes of the fast component of the triplet lifetime at different time spacings from $t = 0$ are plotted. It appears that, after terminating the side illumination up to 40 ms, the amplitude of the fast

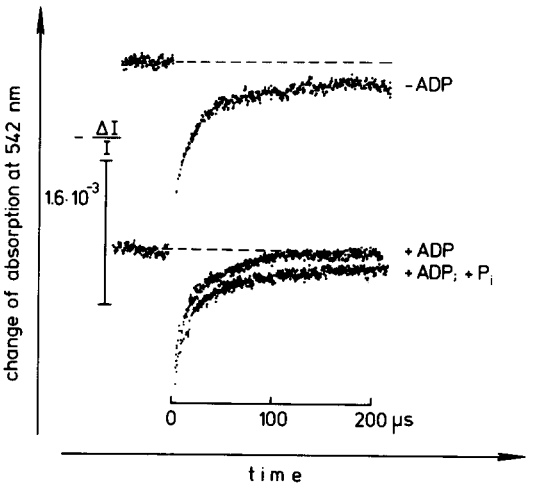

Fig. 3. Effect of ADP and P_i on the absorption changes of the triplet probe bound to membrane-reconstituted BF_1. Labeling was performed in the dark in the presence of ATP (0.9 mol eosin/BF_1).

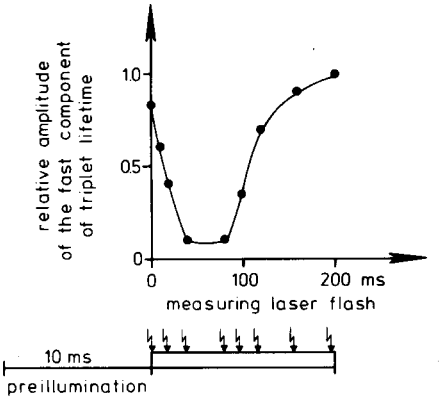

Fig. 4. Time-course of the light-induced conformational change of membrane-bound and labeled BF_1. The change in the relative amplitude of the fast component of the triplet lifetime monitored at different dark times, after membrane energization for 10 ms, is plotted as a function of the dark time.

component decreased to a minimum level of about 10%, which was constant between 40 and 80 ms; after 200 ms, the fast component completely recovered to its original value. We therefore conclude that the conformational change of the labeled and membrane-bound BF_1 appeared after energization of the chromatophores with a half-time of 10 ms, was approx. constant for 40 ms, and disappeared with a half-time of 70 ms.

Measurements of rotational mobility of eosin labeled membrane bound BF_1. Rotational diffusion of eosin-labeled, membrane-bound BF_1 was measured from the dichroic absorption changes of bound eosin, by means of the photoselection technique [13]. For all the experiments, chromatophores reconstituted with BF_1 labeled in the dark and in the presence of ATP (the molar ratio of eosin to BF_1 was one) were used. As shown above, (see Tables II and III), these chromatophores were still very active in ATP synthesis and hydrolysis.

The results are shown in Fig. 5, where the time course of the $A_{\parallel} - A_{\perp}$ difference (upper trace) and that of $A_{\parallel}$ (middle trace) are successively reported. In the lower trace of Fig. 5, the computed fit for the time-course of the absorption anisotropy, is also shown. This trace was obtained by fitting the experimental data by means of a Fourier method, particularly suited to analyze noisy curves with an exponential decay [24]. Kinetic analysis of the data indicates that the total absorbance change, $\Delta A_t = \Delta A_{\parallel} + 2\Delta A_{\perp}$, and the absorption anisotropy parameter $r(t)$ decay monoexponentially with half-times of 286 μs ($\pm$5.8%) and 173 μs ($\pm$9.8%), respectively. The extrapolated initial $r(0)$ value is 0.23 ($\pm$7.5%).

In principle the decay of the absorption anisotropy can be very complex [30]. It can arise from different motions of the transition dipole moment (E-vector) of the dye, which are detectable at different time scales: (a) fast motion of the dye around its binding axis ('librational motion', typically in the picosecond–nanosecond time interval); (b) segmental motions of protein chains which cover the eosin binding sites (in the nanosecond–microsecond time domain); (c) isotropic rotational diffusion of the whole BF_1 complex (in the microsecond time domain); (d) isotropic rotational diffusion of the whole chromatophore (in the micro-

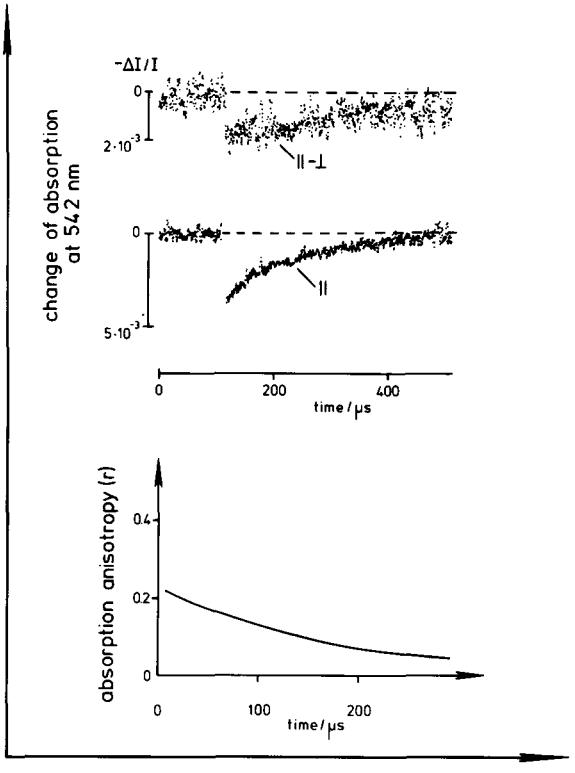

Fig. 5. Time course of the absorption changes of eosin at 542 nm, after excitation with a short laser flash at 532 nm with eosin isothiocyanate bound to BF_1 reconstituted into depleted chromatophores. Labeling was performed as described in the legend of Fig. 3. Upper trace: time-course of the absorption changes for parallel minus perpendicular polarization of the exciting and measuring light. Middle trace: time-course of the absorption changes for parallel polarization. Lower trace: time-course of the absorption anisotropy decay evaluated from the upper and middle traces as specified in Materials and Methods. Curve fitting was performed as described in Materials and Methods.

second–millisecond time scale). We therefore attribute the fast initial loss of anisotropy (not detectable) to either fast librational motion of the dye and/or fast segmental motion of protein chains which cover the eosin binding sites [19]. The remaining decay observed in Fig. 5 can be therefore assigned to isotropic rotational diffusion of the BF_0-BF_1 complex within the chromatophore membrane or of the whole chromatophore. This can be experimentally discriminated by measuring linear dichroism of samples containing the same labeled population suspended in aqueous buffers of different viscosity or of samples containing labeled

chromatophores after glutaraldehyde treatment. The rotational diffusion of the BF_1 complex, when membrane bound, should be in fact nearly independent of the viscosity of the suspending buffer, being mainly regulated by the microviscosity of the membrane environment in which the enzyme complex is embedded and which is at least one order of magnitude higher than that of the aqueous environment [31,32]. In this respect, the change in buffer viscosity should mainly affect the tumbling of chromatophores. Moreover, incubation with glutaraldehyde of the reconstituted labeled chromatophores, should cause crosslinking of the BF_0-BF_1 complex to other membrane components, and thereby prevent a fast rotational diffusion of the enzyme, while the rotational motion due to the whole particle should remain unaffected. When the buffer viscosity was changed by changing the proportion (v/v) of glycerol to the aqueous buffer (we tested samples containing 50, 60 (our usual condition) and 70% glycerol corresponding respectively to 13, 35 and 81cP) the observed fast component of the anisotropy decay was, within the error limits, identical to that shown in Fig. 5; the half-time of a slow component, detectable only in the millisecond time range, increased at increasing buffer viscosity (data not shown). In Fig. 6, trace c, it is clearly shown that the incubation of the chromatophores with the crosslinking agent glutaraldehyde, completely abolished the fast decay of the absorption anisotropy. The fit of the data revealed a slow component with a half-time of 1.86 ms (±28.9%) and an extrapolated initial anisotropy of 0.21 (±18.5%). According to these results, the slow component detectable in the absorption anisotropy decay is most likely due to the rotational motion of the whole chromatophore and the fast relaxation component can be unambiguously attributed to the rotational motion of the whole BF_0-BF_1 complex.

This motion was also measured in the presence of substrates (ADP and P_i) relevant for the enzyme activity. As compared to the experiment shown in Fig. 5, where no substrate was present, addition of ADP and P_i, respectively, did not affect the rotational diffusion of the BF_0-BF_1 complex (Fig. 6, trace a). Only when the chromatophores were preilluminated (for 100 ms) before the measuring laser flash in the presence of both ADP and P_i, the decay of the absorption anisotropy was accelerated (Fig. 6, trace b). Upon glutaraldehyde fixation of the eosin-labeled chromatophores, the addition of substrates did not affect the slow component of the absorption anisotropy with or without preillumination (Fig. 6, trace c). In Fig. 6, the fit of the experimental data points, obtained under the different conditions, is shown. The evaluated rotational diffusion times are 182, 84 and 2400 μs for trace a, b, and c, respectively. The corresponding initial anisotropy values are 0.23, 0.25 and 0.25. It must be noticed that, when the measurements were performed after preillumination but in the absence of substrates, the pattern of the absorption anisotropy decay was identical to that obtained without preillumination in the presence of substrates (Fig. 6, trace a). These results indicate that the rotational mobility of the BF_0-BF_1 complex is increased by a factor of 2 after preillumination of the chromatophore membrane. This increase is directly related to the catalytic action of the enzyme, since it was only observed in the presence of both ADP and P_i.

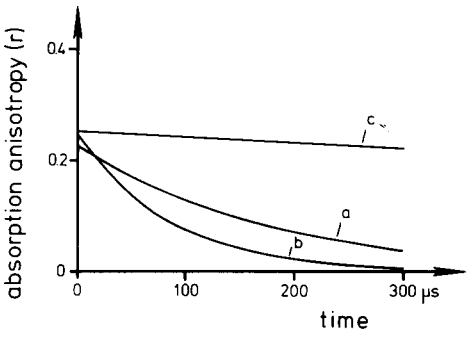

Fig. 6. Time-course of the absorption anisotropy measured in the presence of ADP and P_i, with or without preillumination of the chromatophores. Only the fit of the experimental points is reported. Curve a: as in Fig. 5, lower trace, but in the presence of ADP and P_i. Curve b: as in curve a, after 100 ms of preillumination before the laser flash. Curve c: in the presence of ADP and P_i, with or without preillumination, upon incubation of the chromatophores with glutharaldehyde.

Discussion

The molecular mechanism of the proton translocating ATPase is still a matter of debate, although different approaches were attempted in

order to gain detailed information on energy coupling [29]. The probable reason is perhaps the lack of techniques capable of direct monitoring and time resolving at a molecular level, the early events of energy transduction. The major problem in trying to focus directly on the membrane bound enzyme with spectroscopic techniques, is the impossibility of recognizing a specific endogenous marker on the protein. Labeling with spectroscopic tools, such as triplet probes, can be a suited approach, provided that the newly introduced molecule is specifically localized and is not perturbing the physiological function of the enzyme.

In the present paper we show evidence that the triplet probe eosin can be covalently bound to bacterial coupling factor without altering the properties of the membrane-coupled enzyme as long as the labeling occurs when the protein is still in close interaction with the membrane phase, and in the presence of ATP either in the light or in the dark. Apparently, under these conditions, the catalytic site of the enzyme is protected against inactivation and the ATPase and ATP synthase activities. Bacterial chromatophores reconstituted with this eosin-labeled BF_1 showed rates of ATP synthesis or hydrolysis comparable to those of untreated chromatophores. The Mg^{2+}-dependent ATPase activity of bacterial chromatophores can be light-stimulated. This enhancement, which is oligomycin sensitive, is also detectable in chromatophores reconstituted with labeled BF_1 if the labeling conditions include the presence of ATP.

The lifetime of the triplet probe bound to BF_1 reconstituted into chromatophores is greatly affected when energization of the photosynthetic electron transport system is provided by actinic light for a time interval comparable to that necessary for multiple turnovers of the photosynthetic unit (in the range of 10 ms (for a review, see Ref. 29)). After energization of the chromatophore membrane, in the presence of succinate, the catalytic portion of the enzyme complex undergoes a conformational change, as suggested from the finding that the triplet probe lifetime, covalently bound to the protein, becomes longer or shorter than that measured before illumination, depending on whether labeling had occurred in the dark or in the light. This is interpreted as indicative of an 'opening' of parts of the enzyme upon illumination of the chromatophores, since eosin molecules bound in the dark to BF_1 become less accessible to oxygen (as indicated by the elongation of the triplet probe lifetime) than those bound in the light (as indicated by the shortening of the triplet probe lifetime). The possibility that inner enzymatic sites become exposed to a more hydrophylic environment upon light energization of the chromatophores is consistent with previous results, indicating that certain sulphydryl reagents, such as *N*-ethylmaleimide, inhibit the complex activity only when added to the vesicles in the light and in the absence of ADP [23]. The observed change of the triplet lifetime occurs in parallel with the enhancement of the ATPase activity upon energization; moreover, it is present only when such activation is also detectable, i.d., when the labeled enzyme is functionally reconstituted into the depleted membrane. Oligomycin prevents both the observed change in the triplet lifetime and the enhancement of the light-dependent ATPase activity. More strikingly, when ADP is present during energization of the chromatophores, the change of the triplet lifetime is prevented, in parallel with the stimulation of the ATPase activity, measured under these conditions. The addition of P_i on top of ADP, restores both the enhancement of the ATPase activity and the change in the triplet lifetime. We, therefore, conclude that the membrane energization dependent change in the triplet lifetime of the probe covalently bound to the BF_1-reconstituted chromatophores, is monitoring and time-resolving domain flexibilities of the enzyme complex in its active state. Significantly, the decay pattern of the triplet lifetime after illumination is independent of the extent of the 'phosphate potential', since it is identical in the presence of ATP or of ADP and P_i. Apparently, when the enzyme upon energization is either phosphorylating or hydrolyzing, the same dynamic conformational change is detected with this method. It is also novel that the light dependent conformational change has been time resolved in postillumination. Our results seem to indicate a 'hysteretic' behaviour of at least those portions of the enzyme to which eosin is bound, since the maximal opening of the enzyme is detected after 40 ms of dark (the halftime is about 10 ms). The conformational change remains constant for another 40 ms and

disappears within 200 ms (the halftime is about 70 ms). The persistance of the active state of the enzyme complex in the dark is consistent with previous results obtained by investigating photophosphorylation in postillumination [33]. At present, it is difficult to relate the time-course of the observed conformational change to a concomitant evolution of the energized state of the membrane phase and/or of the bulk-to-bulk phase $\Delta\tilde{\mu}_{H^+}$. The effects observed on the conformational change upon addition of ionophores, uncouplers and inhibitors indicate that in the eosin-labeled chromatophores, antimycin is impairing the light-dependent ATPase activity at a concentration (about 0.5 molecules per reaction center) which usually does not affect the $\Delta\tilde{\mu}_{H^+}$ value; valinomycin and nigericin are effective only at concentrations which severely decrease the total $\Delta\tilde{\mu}_{H^+}$ in untreated chromatophores [28]. It seems, therefore, that in these BF_1-labeled preparations the electron transfer is also necessary to induce a conformational change corresponding to an active state of the enzyme. However, it remains to be established which is the molecular mechanism of the conformational change. Since the 'opening' of parts of BF_1 is detected only in photophosphorylating chromatophores, it can be speculated that this reflects some light-dependent contraction of the BF_0 moiety to form a proton channel, as recently proposed to interpret similar phenomena in chloroplasts [10]. This would cause release of tightly bound nucleotides, as suggested by the inhibitory effect of ADP addition [34] and induce the active state. The 'hysteretic' behaviour of the BF_1 portion in the dark, after preillumination, may also reflect direct regulatory interactions of the catalytic moiety and/or of the membrane sector with redox components of the photosynthetic unit, laterally diffusing within the membrane plane [35].

The results obtained from the photoselection experiments, detecting another type of conformational change, also indicate a strict interaction between BF_1 and BF_0. Among the various rotational relaxation components of the linear dichroism of eosin covalently bound to BF_1 reconstituted into depleted chromatophores, we show that those ranging from 50 to 200 μs are attributable to the rotation of BF_1 in respect to the membrane plane. The rotation of BF_1, when bound to its counterpart BF_0, is much faster than that measured in chloroplasts (180 μs instead of 2 ms [10]). This implies a more fluid environment of the enzyme in the chromatophore than in the chloroplast membrane, and it can be due to the different lipid composition (mainly phospholipid in chromatophores and galactolipid in chloroplasts). Similarly to what has been observed in chloroplasts [10], photophosphorylation substrates plus membrane energization, in the presence of succinate, increase the rotational diffusion of the enzyme complex. The different rotational diffusion times of the BF_1-BF_0 complex in the nonactive (180 μs) and in the active state (80 μs) can be most likely explained by different conformations of the BF_0 part, since this part of the enzyme is embedded in the membrane and should therefore determine the overall rotational mobility of the whole complex. It is interesting, however, to consider that, if BF_0 acts as a proton channel, and the conformational changes we observe with this method are related to a removal of a block to BF_0 to act as proton channel, membrane energization per se is not sufficient for this, since the presence of photophosphorylation substrates is also required.

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