TWO DIFFERENT TYPES OF CONFORMATIONAL CHANGES OF MEMBRANE-BOUND CF1 AS REVEALED BY THE TRIPLET PROBE EOSIN

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Received 9 April 1980
Revised version received 16 April 1980

1. Introduction

The synthesis of ATP in green plants is mediated by the coupling factor CF1 which is bound to the thylakoid membrane via its counterpart CF0 (reviewed [1,2]). It seems well enough established that the two together function as the proton translocating ATP synthase proposed [3]. The molecular details of this action remain to be elucidated.

Conformational changes of CF1 in response to the generation of an electrochemical potential difference of the proton across the thylakoid membrane were detected by various techniques. The membrane was energized either by light [4,5], by artificially induced pH difference [4] or by externally applied electric field [6]. Some of the conformational changes probably reflect the switching of CF1 from an inactive into an active state. Without this, the membrane-bound enzyme does not even function as an ATPase both in broken (class II, [7,8]) and in intact chloroplasts [9]. A full activation of the ATPase activity requires removal of the inhibitory ϵ-subunits of CF1 plus electrochemical energization of the membrane [10]. That the ATP synthase activity (and the concomitant proton conduction) is gated electrically became evident from studies on ATP yield and electric conduction under flashing light [11–14] and under excitation by externally applied electric field [15]. There has been extensive discussion on a catalytic (and energy transducing) role of conformational changes besides the one for activation (reviewed [16]).

Experiments on the exchange of CF1 protons by tritium [3] and on the binding of certain maleimides to the γ-subunit of CF1 [4,17] have suggested that membrane bound CF1 'opens' when the electrochemical potential difference exists and it closes when the potential decays (entrapping as much as 50–100 $^3H^+$/CF1). Another indicator for conformational changes is the exchange of tightly bound nucleotides after energization of the membrane [6,18–20]. One attempt to time resolve conformational changes of CF1 (via fluorescence changes of the label fluorescamine) revealed an upper limit of 45 ms for the switching time [21]. However, from studies on the synthesis of ATP under excitation of chloroplasts with a group of short flashes (fired within 15 ms) it can be inferred that switching occurs in <15 ms even at relatively low values of the electric potential difference in these studies [11]. This is also suggested by the above-cited studies on nucleotide release under externally applied electric field pulses [6].

It seems worthwhile to further characterize conformational changes of CF1 with the aim to specify their role for activation and/or energy transduction. For monitoring conformational changes, we have used eosin isothiocyanate as covalent label for CF1. The triplet lifetime of eosin is markedly enhanced when this dye is bound to proteins. This has been used (reviewed [22]) to study the rotational diffusion of proteins in membranes.

We have observed that eosin-SCN (when bound to CF1) indicates two different types of conformational changes of the membrane bound coupling factor:

(1) An opening of CF1 in response to membrane energization (via a shortening of the triplet lifetime of bound eosin);
(2) A contraction of those parts of the enzyme which are exposed to the membrane viscosity in response to ADP and light (via the rotational correlation time of eosin–CF1).

2. Materials and methods

Spinach was purchased from the local market and prepared by standard procedures [23]. Eosinisothiocyanate (eosin-SCN) was prepared as in [24]. Chloroplasts were labeled with eosin-SCN in a medium containing: chl, 50 μM; NaCl, 50 mM, $MgCl_2$, 5 mM; tricine (pH 8.25) 50 mM; sucrose, 250 mM; pyocyanin, 30 μM; final vol. 20 ml. Light incubation was performed under stirring, with the reaction vessel in a thermostated (20°C) water bath under illumination with white light (0.7 W/cm^2). Eosin-SCN was dissolved in the same buffer (immediately before use!) to 4 mg/ml and added to the chloroplast suspension to give the indicated final concentration. The incubation procedure is illustrated in fig.1.

After incubation the chloroplast suspension was centrifuged at 10 000 × g for 10 min and washed 3 times with the incubation buffer. Labeled CF1 was isolated as in [25]. Protein was determined by the Lowry method [26] and the amount of bound eosin was determined spectrophotometrically using $\epsilon = 8.3 \times 10^4\ M^{-1}.cm^{-1}$ for eosin at 532 nm [24].

Covalent binding of eosin was checked by adding SDS (1% final conc.) to the isolation buffer [25], heating the sample for 10 min to 60°C then applying the sample to a Sephadex G-25 column (1.5 × 20 cm) which was equilibrated with the same buffer. CF1 was eluted in the void volume. At least 85% of eosin associated with CF1 was covalently bound according to this protocol.

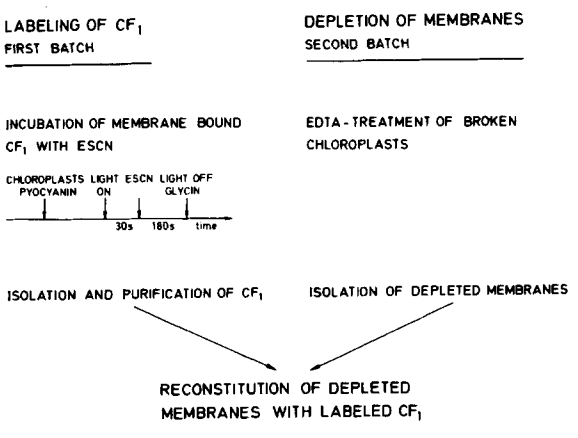

Fig.1. Procedure for the preparation of chloroplasts which are specifically labeled on CF1 by eosin-isothiocyanate (ESCN). 'Light-labeling' is shown; the procedure for 'dark labeling' is the same, except for the light.

CF1 depletion of chloroplasts as well as reconstitution of labeled CF1 into CF1-depleted membranes was performed (with slight variations) as in [27]. After reconstitution the chloroplasts were centrifuged at 7000 × g for 7 min and resuspended in a small volume of the reconstitution buffer [27]. The rate of cyclic photophosphorylation was measured as in [29] and the Ca^{2+}-ATPase activity of the isolated CF1 as in [30]. The principles of flash spectrophotometry and its instrumentation are detailed in [31]. The standard sample for the flash spectrophotometer experiment contains: bovine serum albumin (supergrade), 1 mg; EDTA (pH 7.9) 0.5 μM; $MgCl_2$, 5 μM; tricine (pH 8), 50 μM, NaCl, 50 μM; CF1 reconstituted chloroplasts, at 20 ng chl.; in 1 ml final vol. ADP (3 μM) and phosphate (pH 8, 2 μM) were added as indicated. For the measurements of the rotational diffusion of the reconstituted and dark labeled CF1 the sample was deoxygenated by a stream of dry and cleaned argon which was flushed over the sample surface for 5 min. The sample was filled into a 1 cm optical cell with a thermostated glass mantle sealed against oxygen.

The optical samples were excited with a pulse from a Q switched Nd-YAG Laser (Laser Ass.) which was frequency doubled (532 nm). Pulse duration was 20 ns and pulse energy ~10 mJ. The excitation pulse saturated usually 10% of the eosin molecules in the sample. While the laser beam was intrinsically polarized (E-vector vertical) the measuring light was linearly polarized by rotatable Glan Thomson prism. Flash-induced absorption changes were recorded with a Biomation transient recorder and averaged on a digitally interfaced H. Nicolet (1072) averaging computer.

To follow the rotational diffusion of labeled CF1 we have applied the technique of photosection as in [34] (reviewed [22]). The geometrical features of polarized excitation and observation of an initially isotropic chloroplast suspension are illustrated in fig.4D. With $\Delta A_\parallel$ and $\Delta A_\perp$ being the observed absorption changes under parallel and perpendicular polarization, respectively, the following observables will be interpreted: $\Delta A_t = \Delta A_\parallel + 2\Delta A_\perp$ (showing the depletion and repopulation of the eosin ground state without interference of rotational diffusion) and $r = (\Delta A_\parallel - \Delta A_\perp)/\Delta A_t$ (the rotational anisotropy parameter, showing rotational diffusion of excited eosin without interference of the repopulation of the ground state).

3. Results and discussion

Due to the high reactivity of the SCN-group for amino- and sulfhydryl groups of proteins [31] it was to be expected that incubation of thylakoids with eosin-SCN produces labeling at many different sites. Table 1 shows the amount of eosin-SCN bound to thylakoid membranes and specifically to CF1, respectively. All samples were incubated for 180 s either under illumination or in the dark. The number of eosin molecules bound to all available sites (left column) is given in relation to 860 chl for better comparison. (There is ~1 CF1/860 chl [32].) The number of eosins specifically bound to CF1 is given in the right column. It is evident that eosin-SCN binding to CF1 and to the other sites is greatly facilitated when the membrane is energized by light. (Reversal by uncouplers NH_4Cl plus gramicidin was observed.) This can be interpreted as follows:

> The generation of an electrochemical potential difference of the proton across the membrane causes conformational changes of CF1 and also of other proteins thus opening additional binding sites for the attack by eosin-SCN.

The large number of binding sites on proteins other than CF1 made it necessary to isolate labeled CF1 and to reconstitute it into freshly depleted membranes. This produced specifically labeled samples. The phosphorylation activity of chloroplasts reconstituted with labeled CF1 was affected differently depending on whether labeling had occurred in the dark or in the light. One example: at a load of 1.3 eosins/CF1 (adjusted by higher eosin-SCN concentration during dark than during light incubation) the rate of cyclic ATP synthesis was 100% of the control if incubation occurred in the dark but only 55% of it occurred in the light. The absolute figure for the control (reconstituted with unlabeled CF1) was 328 μM ATP/mg chl . h under excitation at 0.1 W/cm^2 and with pyocyanin, 30 μM as cofactor. (In both cases the Ca^{2+}-ATPase activity was only inhibited by 10%.) This can be interpreted as follows:

> Those binding sites on CF1 which become accessible after energization of the thylakoid membrane differ from those, which are accessible already in the dark, by their greater influence on ATP-synthesis.

More detailed information on the binding sites and on the effects of labeling will be subject of a forthcoming paper.

Table 1
Incorporation of eosin-SCN into the membranes of broken chloroplasts (left column) and into CF1 in particular (right column) as function of [eosin-SCN] and illumination during the incubation period (cf. fig.1)

Incubation	Eosins bound to membrane/860 Chl	Eosins recovered with CF1 after isolation
Dark		
160 μM	31	2.5
54 μM	11	1
Light		
160 μM	79	6
54 μM	13	3

Fig.2 shows ΔA_{543} observed under excitation of an aqueous suspension of isolated CF1–eosin-SCN with a short laser pulse. The absorption changes reflect the ground state depletion of eosin and its subsequent repopulation from the triplet state. These absorption changes are strictly paralleled by those around 630 nm which characterized the triplet absorption of eosin.

It is apparent from a comparison of the two traces that the triplet lifetime of eosin is drastically increased when labeling has occurred in the light. This observation is interpreted as follows:

> The additional binding sites for eosin-SCN on CF1 which become accessible only after energization of the thylakoid membrane are better protected against the triplet quencher dioxygen.

Fig.3 shows the absorption changes of eosin after reconstitution of light labeled CF1 into pre-depleted membranes. The signal to noise ratio in fig.3 is necessarily worse than in fig.2 due to lower concentration of labeled CF1 in the absorption cell. The flash burst artefact is also worse for the same reason. (It is difficult to avoid under excitation at 532 nm and observation at 543 nm.) Underlying signals due to intrinsic absorption changes of thylakoids, mainly due to electrochromism of carotenoids [33], are comparatively small ($< +10^{-4} = -\Delta I/I$). It is apparent from inspection of fig.3 that the lifetime of the eosin triplet state (light labeled into CF1) is shortened if the thylakoid membrane is energized by light. (The reversal by uncoupler has been observed!) This can be interpreted as follows:

> Even light labeled CF1 can carry out conformational changes in response to the energization of the thylakoid membrane thus exposing inside located

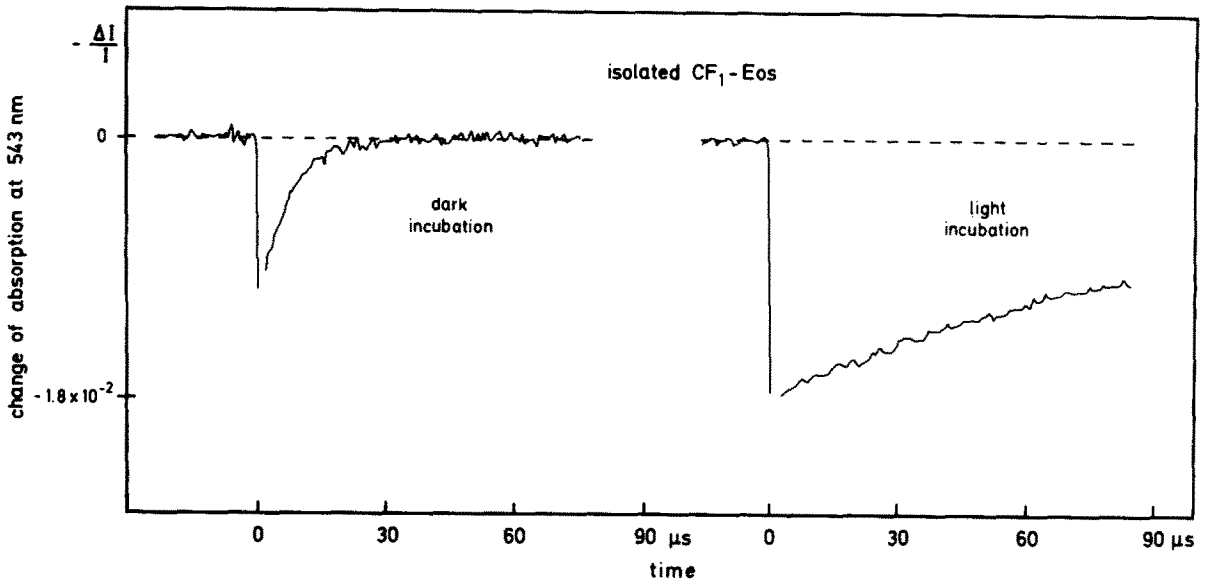

Fig.2. Time course of the ΔA_{543} of eosin (ground state depletion) with isolated CF1–eosin-SCN upon excitation with a short loser flash at $t = 0$. Left, light labeled CF1; right, dark labeled CF1. Air saturated buffer, 1.3 eosin-SCN/CF1 for both traces.

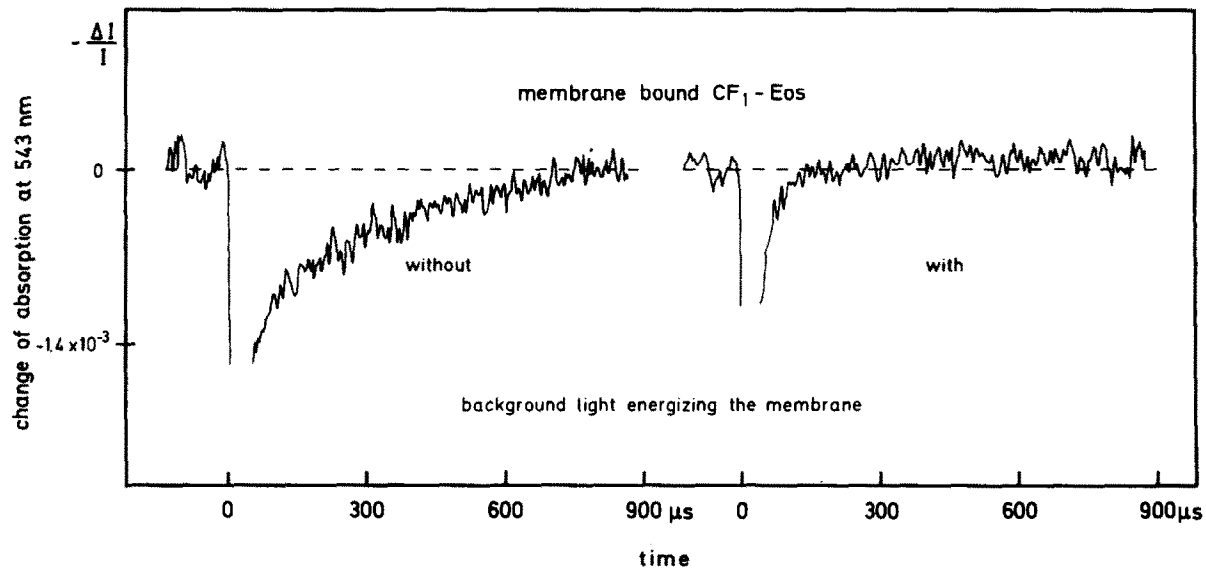

Fig.3. Time course of the ΔA_{543} eosin (ground state depletion) with membrane-bound CF1 which was 'light labeled' with eosin-SCN before reconstitution. Excitation with a short laser flash. Left, without energization; right; with energization of the membrane by strong continuous light ($\lambda > 660$ nm). O_2-depleted buffer. 1.3 eosin-SCN/CF1.

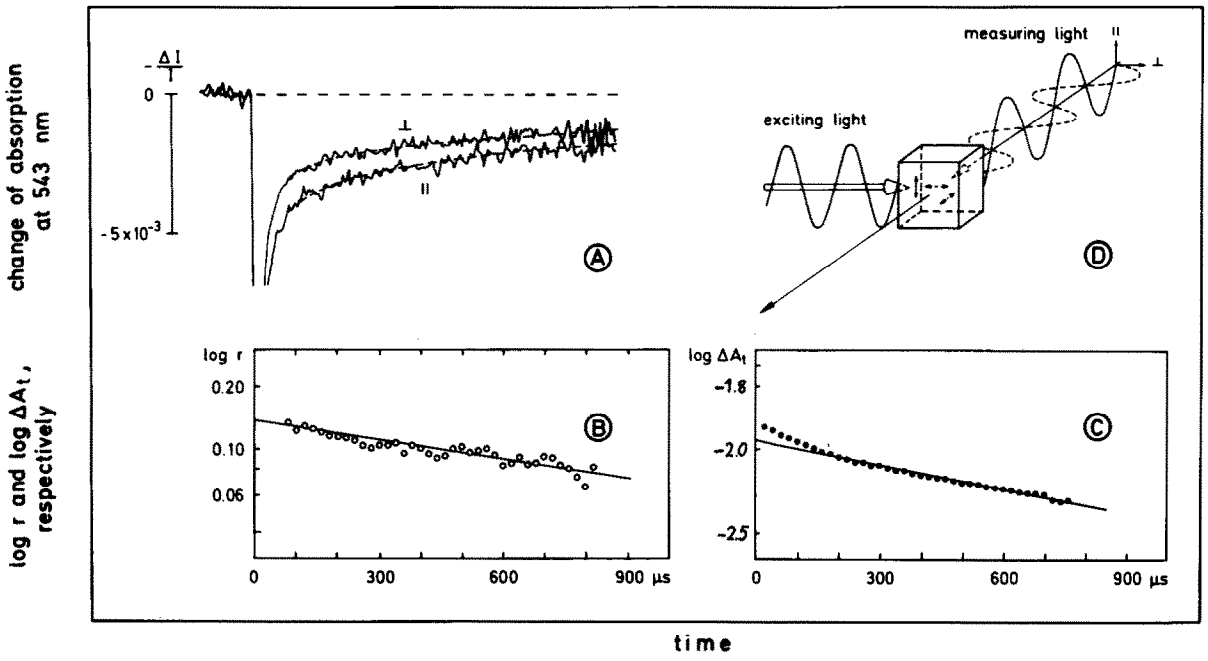

Fig.4. Linear dichroism of the ΔA_{543} of eosin in a photoselection experiment with membrane bound CF1, which was 'dark labeled' with eosin-SCN before reconstitution. (A) Time course of the absorption changes; (B) time course of the *r*-parameter (half-log plot); (C) time course of the total absorption changes ($\Delta A_{\parallel} + 2\Delta A_{\perp}$, half-log plot); (D) geometry of a photoselection experiment.

eosins to the triplet quencher oxygen. The absorption changes of eosin, via their triplet lifetime (light incubation), are therefore a rapid kinetic probe for the opening and closing of CF1.

Results on the rotational diffusion of eosin-labeled membrane bound CF1 are shown in fig.4 and in table 2. The decay of the *r*-parameter can be very complex already in rotating bodies with a fixed chromophore [35]. Even more in the present experiments where rotation of the chromophore in the protein, of the protein within the membrane and of the membranes in the suspending medium have to be considered. In studies with labeled CF1 suspended in a highly viscous solvent we found rapidly relaxing components attributable to residual motion of bound eosin (in preparation). On the other hand we have measured the rotational relaxation of thylakoids in the suspending medium and observed half times of some 100 ms (via the linear dichroism of P700, Graef and Junge, unpublished). Therefrom it is clear that the moderately fast rotational relaxation documented in fig.4B is due to rotational diffusion of CF1 in the thylakoid membrane. It is noteworthy that this relaxation showed a distinct temperature transition (in preparation).

Table 2

The rotational relaxation time of eosin-labeled CF1 ($\tau_{rot.}$) under variation of parameters relevant for photophosphorylation: background light (in the presence of PMS, driving cyclic electron transport and proton pumping), ADP + P and the uncoupler NH_4Cl (for concentrations see section 2)

Background light	ADP + P_i	NH_4Cl	$\tau_{rot.}$ [µs]	T [°C]
–	–	–	1670	6
+	–	–	1730	6
–	+	–	1090	8
+	+	–	580	8
+	+	+	1100	8

It is most interesting that the decay of the *r*-parameter is dependent on phosphorylation parameters as ADP (and P), light and the uncoupler NH_4Cl. This is shown in table 2. Light alone is ineffective, contrary to its effect on the triplet lifetime of 'inside bound' eosin. However, addition of ADP + P shortens the relaxation time, and then light shortens it further.

The latter effect is reversed upon addition of the uncoupler. It is noteworthy that these effects were observed with 'dark labeled' CF1 which remains active in ATP-synthesis. This can be interpreted as follows:

Light labeled CF1–eosin-SCN besides undergoing conformational changes of the 'opening–closing type' undergoes conformational changes which change that part of the enzyme exposed to the microviscosity of the thylakoid membrane, perhaps via CF0.

4. Conclusions

A technique to monitor conformational changes of the membrane-bound coupling factor of photophosphorylation (CF1) is presented. Covalent labeling of the membrane-bound CF1 with eosin-SCN either in the light ('inside' bound eosin-SCN) or in the dark ('outside' bound eosin-SCN) prolongs the triplet lifetime of eosin. When the thylakoid membrane is energized by a (light generated) electrochemical potential difference, 'inside' bound eosin molecules react with a drastic shortening of their triplet lifetime. This reflects an 'opening' of CF1 (better access of O_2 to eosin), which was also apparent from other experimental approaches (section 1). The advantage of the eosin technique over others (except the fluorescamine technique) is its potentially high time resolution for the opening of the CF1 structure. A possible disadvantage is the inhibition of 'inside' labeled CF1 to act as an ATP synthase. This may cause 'ill' switching of CF1 as we observed for CF1, which was 'inside' modified by orthophenyldimaleimide [37].

Another type of conformational changes of membrane-bound CF1 became apparent from photoselection experiments on the rotational diffusion of eosin bound to CF1. It is noteworthy that the rotational diffusion can be measured with 'outside' bound eosin-SCN, which leaves CF1 catalytically active. Among the various rotational relaxation components of the linear dichroism of eosin at least those ranging between 100 μs and 10 ms are attributable to rotation of CF1 in the membrane. (They are not attributable to motion of the label within the protein or to rotation of chloroplasts in the suspension.) It is a new observation, that CF1 carries out rotational diffusion at ~2 ms (6°C), which implies a very high microviscosity of the thylakoid membrane. It is also novel, that ADP and illumination increase the velocity of its rotational diffusion. The latter shows that ADP and energization affect the size of those parts of CF1 (and CF0) which are exposed to the microviscosity of the thylakoid membrane.

It may be speculated that the tightly bound nucleotides plus energization of the thylakoid membrane are gating the proton channel through CF0 (section 1). In view of the evidence for a pore-type action of CF0 for protons it is also conceivable that the acceleration of the rotational diffusion of CF1 reflects the removal of a block to the contraction of the (3 or 6) subunits of CF0 to form a pore. The methods presented here could test this possibility.

Acknowledgements

We wish to thank Ilse Columbus and Allison McGeer for helping during the experiments and Paul Schnetkamp for critical comments on the manuscript. Special thanks to Ilse Columbus for many years of collaboration and sympathy. The experiments were carried out when we were working at the Max-Volmer-Institut, Technische Universität Berlin. Margret Offermann did the drawings. Financial support came from the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

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