Conformational Changes of the Membrane-Bound ATPase of Bacterial Chromatophores Revealed by Fluorescence Changes of Fluorescamine-Labelled Coupling Factors

Peter Gräber and Sathamm Saphon *

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin

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Chromatophores Coupling Factors, Fluorescence-Labelling, Conformational Changes

The solubilized coupling factor (F_1) of *Rps. sphaeroides* chromatophores was allowed to react with fluorescamine which led to a fluorescence labelled F_1. After reconstitution with the depleted membranes the fluorescence-labelled F_1 was shown to restore photophosphorylation in continuous light in a similar way to the non-labelled F_1. In parallel, a decrease of the fluorescence emission of the labelled and reconstituted coupling factor was observed. The solubilized and labelled F_1 showed also a fluorescence decrease as the polarity of the medium was increased. In single turnover flashes the fluorescence change was found to be inhibited by an uncoupling agent such as FCCP. The kinetics of the change were sensitive to phosphorylating agents and to an "energy transfer inhibitor" such as venturicidin.

It is suggested that the observed fluorescence changes reflect conformational changes of the ATPase enzyme complex.

Introduction

In photosynthesis phosphorylation is coupled to light-dependent electron transport by a membrane-bound enzyme which also shows a latent or manifest ATP-hydrolyzing activity. This enzyme can be removed from the native membranes or reconstituted back with the depleted ones with a parallel loss or restoration of phosphorylating activity [1–4].

It is well accepted that the free energy for ATP-synthesis is conserved in a transmembrane electrochemical proton difference (ΔpH and $\Delta\varphi$). The release of the energy by a proton efflux via the ATPase pathway was assumed to be coupled with ATP formation [5–7]. The mechanism of coupling *within* the ATPase is subject to different hypotheses. A direct participation of H^+ via protonations at the catalytic site of the ATPase has been proposed by Mitchell [8]. An indirect participation of H^+ was proposed by Boyer [9, 10] and Slater [11]. Protonations at one site of the enzyme induce conformational changes which lead to changes in binding affinities for nucleotides at the catalytic site.

Different attempts have been made to detect conformational changes of the ATPase enzyme-complex, *e.g.* incorporation of ^3H [12, 13], inhibition of phosphorylation after preincubation with enzyme inhibitors [14, 15], and energy-dependent exchange of adenine nucleotides which are tightly bound to the coupling factor [16–20]. One promising method to investigate these phenomena seemed to be the fluorescence labelling of the coupling factor with fluorescamine as it has been used earlier in chloroplasts [21].

Using chromatophores of *Rps. sphaeroides* it is shown in this work:

1.) Reconstitution of the fluorescamine-labelled coupling factor, F_1, to depleted membranes restores phosphorylation in continuous light.

2.) The fluorescence of the reconstituted F_1 is quenched upon energisation of the membrane either by continuous light or by single turnover flashes.

3.) The kinetics of the flash induced fluorescence change responds to phosphorylation.

4.) In accordance with results obained in chloroplasts [21] it is concluded that the observed fluorescence changes reflect conformational changes of the re-attached coupling factors [38].

5.) The conformational changes detected by the observed fluorescence changes are not generated by the release of energy from the electrochemical proton gradient.

* Present address: Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

Abbreviations: FCCP, carbonylcyanide-p-trifluormethoxyphenylhydrazone; P_i, inorganic phosphate; BSA, bovine serum albumin.

Requests for reprints should be sent to Dr. P. Gräber, Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin, Straße des 17. Juni 135, D-1000 Berlin 12.

Materials and Methods

Chromatophores from *Rps. sphaeroides* were prepared from anaerobically grown cells as has been described in ref. [22]. Techniques similar to those described in [3, 4, 23] have been applied to resolve the chromatophore coupling factor, F_1. Chromatophores were suspended in a medium containing 50 mM tricine pH 7.2, 1 mM ethylene-diamine-tetra-acetic-acid (EDTA), 10% sucrose at a concentration of about 0.2 mM bacteriochlorophyll, sonicated at 4 °C for 4 bursts of 30 seconds in a Branson B 12 ultrasonic desintegrator (time between the bursts 60 seconds), and stirred for 1 hour in the dark at room temperature. After centrifugation for 2 hours at $140\,000 \times g$ the sediment of partially resolved chromatophores was washed once and resuspended in the above medium (with omission of EDTA). The supernatant was used either directly as a source of coupling factor or after concentrating the protein by ultrafiltration (Amicon, Diaflo XM100A) without further purification.

The fluorescence labelling of the protein with fluorescamine [21, 24] was carried out as follows. A solution of fluorescamine in acetone (15 mM) was added in 10 μl aliquots to about 10 ml protein solution (0.2 – 0.7 mg protein per ml) while mixing very rapidly on a vortex-mixer at room temperature (about 0.6 μmol fluorescamine was added per mg protein). The reaction was complete within a few seconds. Unsoluble reaction products, when observed, were removed by centrifugation.

Reconstitution of the coupling factor with the depleted membranes was performed in the dark after addition of $MgCl_2$ (final concentration 10 mM) for 30 – 45 min at room temperature as described in [23]. All experiments were carried out using chromatophores (treated or untreated) in within 36 h of preparation.

ATP-synthesis in continuous light was measured using the ^{32}P-method as described in refs [22, 25]. (Succinate was not present in the reaction mixture.) ATP-hydrolysis was measured at 30 °C as described in ref. [39]. Protein concentration was determined according to Lowry *et al.* [26], bacteriochlorophyll concentration according to Clayton [27].

Optical measurements were performed using a flash spectrophotometer and a signal averaging system as described in ref. [28]. Photosynthesis was excited repetitively with xenon-flashes (20 μs duration, wavelength 665 nm, flash frequency 1/15 Hz). Fluorescence of the labelled F_1 was excited at 367 nm (light intensity ≈ 800 erg/cm^2·s) and the emission measured at an angle of 90° at 497 nm. The photomultiplier was shielded from flash light by a 585 nm cut-off filter (Balzers), a 4 mm GG 385 (Schott) and a 3 mm BG 28 (Schott). Electrical bandwidth of the apparatus was dc – 160 Hz. The capillar cuvette (1 mm opical pathway) used contained usually a solution with 10% sucrose, 50 mM KCl, 8 mM $MgCl_2$, 50 mM tricine pH 8, 0.2 mM bacteriochlorophyll and if indicated 0.5 mM P_i and 0.66 mM ADP.

The fluorescence spectra of the labelled F_1 and of the reconstituted chromatophores have been measured with a Perkin-Elmer MPF 4 fluorescence spectrometer. The fluorescence was excited at 367 nm ($\Delta\lambda \approx 7$ Å), the emission measured at an angle of 90°. The 1 cm cuvette contained the same solution as described above except the bacteriochlorophyll concentration was 3.6×10^{-6} M. The spectra were not corrected for inner filter effects *i. e.* for the absorption of the membrane pigments. Continuous ligh illumination ($\lambda \geqq 665$ nm, Schott RG 665) was applied from the top of the cuvette via a light guide.

Results

Table I shows the rate of ATP-synthesis of native chromatophores, of chromatophores partially depleted of coupling factor, and of chromatophores reconstituted with the labelled or the unlabelled F_1.

Table I. Rate of ATP-synthesis induced by continuous light in chromatophores under various conditions and rate of ATP-hydrolysis of the fluorescence labelled and unlabelled coupling factor extract. For further details see text and Materials and Methods.

Conditions	Rate of ATP-synthesis [μM ATP/μM BChl·min]
control chromatophores	2.9
resolved chromatophores	0.8
chromatophores reconstituted with unlabelled coupling factor	1.9
chromatophores reconstituted with labelled coupling factor	1.7
Conditions	**Rate of ATP-hydrolysis [μM P_i/mg protein·h]**
unlabelled coupling factor	1.6
labelled coupling factor	2.5

Though no complete extraction could be achieved in this preparation and also no complete restoration of the control rate of phosphorylation could be observed after reconstitution, the rate obtained with chromatophores reconstituted with the labelled F_1 was shown to be only about 10% less than that obtained by reconstitution with the unlabelled F_1. The labelling of the coupling factor extract was performed with excess fluorescamine (see Materials and Methods). It is, therefore, unlikely that the reconstitution with the extract containing labelled F_1 is due to non-labelled F_1 which had not reacted with fluorescamine. Furthermore, since extraction and reconstitution of F_1 was shown to have no effect on the permeability of the chromatophore membrane [23] the increase in the rate of phosphorylation after reconstitution suggested that the rebound coupling factors were indeed active in ATP-synthesis.

The rates of Mg^{2+} dependent ATP-hydrolysis of the solubilized coupling factor before and after labelling with fluorescamine are also shown in Table I. The rates were in both cases very low as was found by other authors [3, 4, 29] and the change due to labelling did not seem significant. It should be noted that ATP-hydrolysis catalyzed by the coupling factor of spinach chloroplasts was found to be inhibited by fluorescamine treatment. However, the labelling with fluorescamine and the ATPase activity in those experiments were performed after activation by heat or dithiothreitol [30, 31].

Fig. 1 shows the fluorescence emission spectrum of soluble F_1 labelled with fluorescamine at pH 8. Changes of pH between pH 9 and pH 7 resulted in only small changes of the fluorescence intensity (not shown). However, addition of dioxane drastically increased the fluorescence intensity. Curves B and C were obtained at a mole fraction of dioxane of $x = 2\%$. and $x = 9\%$ respectively. This increased fluorescence may result from the decreased polarity of the suspension medium.

Fig. 2 shows the uncorrected fluorescence emission spectrum (excitation at 367 nm) of chromatophores reconstituted with labelled F_1. Comparison with the results from Fig. 1 suggested that the fluorescence emission spectrum interfered with the absorption spectrum of the chromatophore pigments which are mainly carotenoids in the range of wavelengths considered ([32], see also Discussion). Continuous light illumination ($\lambda \geqq 665$ nm) induced a decrease of fluorescence over the whole range of wavelengths (435 – 510 nm). Similar changes have been observed in spinach chloroplasts which have been reconstituted with fluorescamine-labelled coupling factors [21]. The observed fluorescence emission originated from labelled coupling factors which were reattached to the membrane and

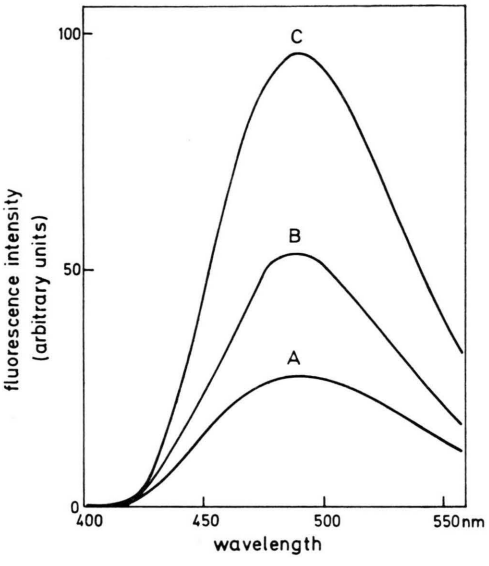

Fig. 1. Fluorescence emission spectrum of the crude coupling factor extract labelled with fluorescamine. A) suspended in H_2O, 2×10^{-3} M tricine, pH 8; B) as in A) but with addition of dioxane, mole fraction 2%; C) as in A) but with addition of dioxane, mole fraction 9%. Fluorescence was excited at 367 nm. Details see text and Materials and Methods.

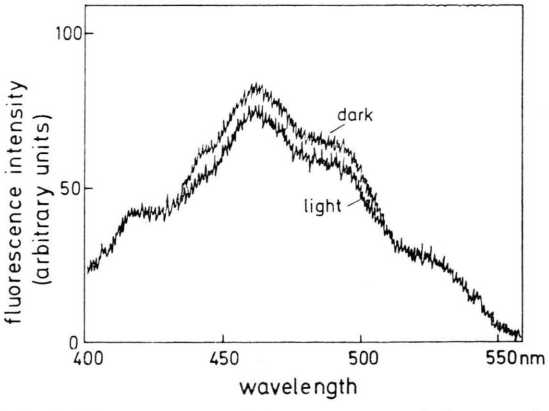

Fig. 2. Fluorescence emission spectrum of chromatophores reconstituted with labelled F_1 in the dark and with actinic illumination. Details see text and Materials and Methods.

also from those which were still soluble in the suspension medium. However, as is shown below, the decrease of fluorescence induced by continuous light resulted only from coupling factors attached to the membrane.

In Fig. 3 the fluorescence changes (measured at 497 nm) induced by single turnover flashes are shown under different conditions. Trace c shows the fluorescence decrease obtained with chromatophores reconstituted with labelled coupling factor. Addition of the uncoupler FCCP abolished the change completely (trace d). Resolved chromatophores alone (trace a) and the labelled coupling factor alone (trace b) showed no flash-induced fluorescence change.

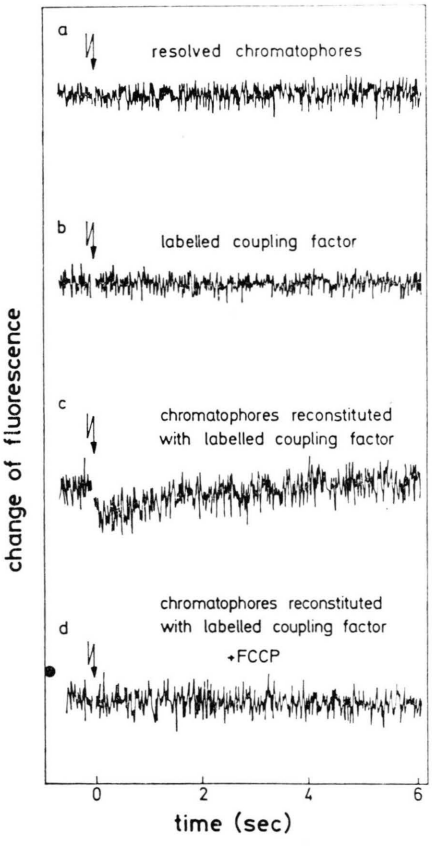

Fig. 3. Change of fluorescence measured at 497 nm induced by single turnover flashes under different conditions a) resolved chromatophores, b) labelled coupling factor, c) resolved chromatophores reconstituted with labelled coupling factor, d) resolved chromatophores reconstituted with labelled coupling factor in the presence of FCCP (10^{-6} M). The traces have been obtained from an average of 10 flashes, frequency 1/15 Hz. For further details see text and Materials and Methods.

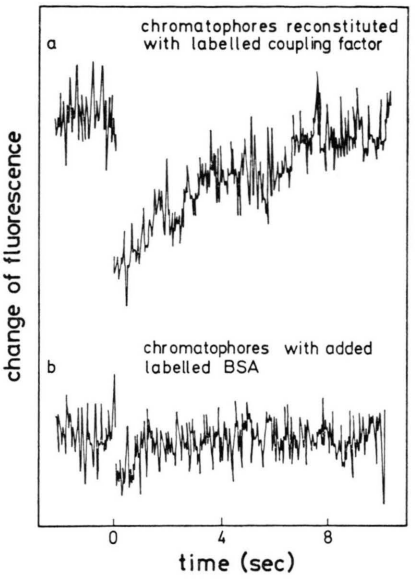

Fig. 4. Change of fluorescence at 497 nm, induced by single turnover flashes a) chromatophores with labelled coupling factor b) chromatophores with labelled BSA. The traces have been obtained from an average of 50 flashes, flash frequency 1/15 Hz. Details see text.

The observed intensity changes of fluorescence may result from an absorption change of the chromatophore pigments. Therefore, fluorescing bovine serum albumin (BSA labelled with fluorescamine similar as described for F_1, see Materials and Methods) was added to give the same fluorescence intensity. Fig. 4 shows the flash induced fluorescence change of chromatophores reconstituted with labelled F_1 (trace a) and of chromatophores with added, labelled BSA (trace b). As can be seen there exists a small artifact signal which may result from the overlapping absorption change of the membrane pigments.

Fig. 5 shows the flash induced fluorescence change obtained in the absence of any phosphorylating agents (trace a), in the presence of ADP and P_i (trace b) and in the presence of ADP, P_i and the ATPase-inhibitor venturicidin [23] (trace c).

Under phosphorylating conditions the relaxation of the signal was accelerated (half life time $\approx$ 0.8 s) compared with that under non-phosphorylating conditions (half life time $\approx$ 1.7 s) and this acceleration was reversed by venturicidin which was previously shown to inhibit phosphorylation [23].

Although accurate analysis of the decay of the fluorescence changes was difficult in view of the

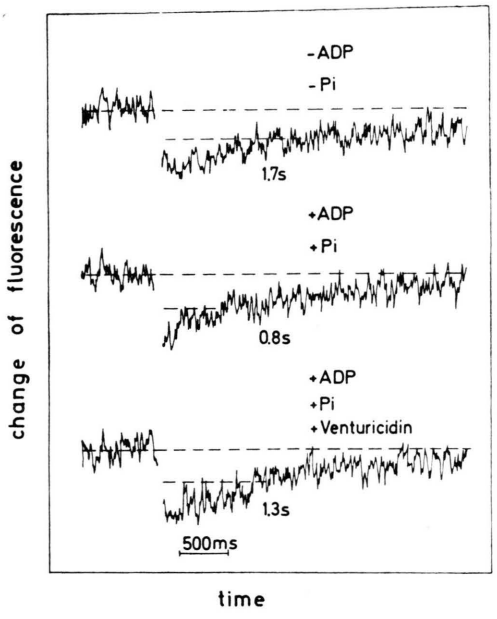

Fig. 5. Change of fluorescence measured at 497 nm using a suspension of chromatophores reconstituted with labelled coupling factor excited by single turnover flashes under different conditions: a) in the absence of ADP and P_i, b) in the presence of ADP and P_i, c) in the presence of ADP and P_i and venturicidin. The traces have been obtained from an average of 120 flashes, flash frequency 1/15 Hz. A fresh sample was used after every 20 flashes. The concentrations of ADP, P_i and venturicidin were 0.66 mM, 0.5 mM and 1 μg/ml respectively. For further details see text and Materials and Methods.

low signal to noise ratio it could be shown here that the relaxation kinetics of the change were sensitive to phosphorylating agents. Similar effects have also been observed earlier for the flash-induced carotenoid shift in reconstitution experiments with the non-treated coupling factor extract [23]. A correlation between both events will be reported elsewhere.

Discussion

The results presented in this work suggested that extraction of the chromatophore coupling factor, F_1, subsequent labelling of F_1 with fluorescamine and rebinding of the fluorescence labelled F_1 to the depleted membranes was possible without significant loss of activity compared with that of the non-labelled factor. Since it was shown that no permeability changes occur in chromatophores after extraction and recondensation of F_1, the recondensed F_1 must, therefore, be functionally active in forming ATP. This was not necessarily true in the case of chloroplasts [33]. After reconstitution of the labelled F_1 to the depleted membranes a fluorescence decrease induced either with continuous light or single turnover flashes (Figs 2 and 3) was observed. This change was sensitive to an uncoupler such as FCCP (Fig. 3) and to phosphorylating agents (Fig. 5).

The question arises as to the origin of the light-induced fluorescence decrease. Two possibilities should be considered:

1.) The fluorescence decrease was trivially due to an overlapping absorption increase of the membrane pigments. Over the range of wavelengths considered (435 – 510 nm) the main candidates are the carotenoids. The shape of the uncorrected fluorescence emission spectrum in Fig. 2 suggested an interfering absorption by those pigments: Minima of fluorescence bands were observed where maxima of carotenoid absorption bands occurred. The carotenoids, however, do not show a light-induced absorption increase but rather an absorption band shift [34]. If this is the case an alternating fluorescence increase and decrease at the corresponding wavelengths should be expected. This, however, has not been observed. The light-induced carotenoid band shift which occurred, could be masked by the larger fluorescence decrease due to other types of reactions of the labelled coupling factors. Another argument against this possibility was provided by the fact that only a small flash-induced fluorescence quenching has been observed with a suspension of chromatophores with added fluorescence-labelled BSA measured under similar conditions (Fig. 4).

2.) The fluorescence decrease reflected a change associated with the labelled F_1 which has been reattached to the membrane and which may be due, for example, to a light induced electric field across the membrane [34, 35], to changes in pH or to changes in polarity in the micro-environment of the fluorescing groups.

a) In the case of a change due tot he light-induced electric field (delocalized or localized in the environment of F_1) the fluorescing groups of the rebound and labelled F_1 monitored changes of the electric field, in a similar way as the carotenoids [34 – 37]. This possibility cannot be as yet ruled out. However, preliminary experiments showed that the relaxation kinetics of the flash-induced carotenoid shift did not match completely with those of

the fluorescence decrease. A detailed analysis will be reported elsewhere.

b) A fluorescence decrease due to changes in pH seemed unlikely because of the small effect of pH on the fluorescence of the soluble and labelled F_1, and, furthermore because of the small flash-induced pH-changes in the suspension medium (strongly buffered solutions were used in the experiments).

c) A fluorescence decrease due to changes in polarity of the micro-environment of the fluorescing groups should finally be considered. Since the fluorescence intensity of the soluble and labelled F_1 varied strongly with the polarity of the suspension medium (Fig. 1) this possibility did not seem unlikely. Assuming that the fluorescence properties of the soluble and labelled F_1 were similar to those of the re-bound F_1, a light-induced fluorescence decrease may indicate a change of the polarity in the environment of the fluorescing groups towards higher values. This could be accounted for, for example, by changes of the conformation of the reconstituted coupling factor leading to an exposure of the fluorescing groups to an environment of higher polarity (*e. g.* into the aqueous phase).

This interpretation has been first proposed by Kraayenhof and Slater [21] for the light-dependent fluorescence changes occurring in chloroplasts after reconstitution with fluorescamine-labelled coupling factor, CF_1. If this interpretation is correct one may conclude that conformational changes of the coupling factor of bacterial chromatophores occur upon energisation of the membrane either by continuous light or by single turnover flashes. These conformational changes of F_1 are observed under phosphorylating as well as under non-phosphorylating conditions. Under phosphorylating conditions the relaxation of the flash-induced conformational changes was accelerated and the acceleration was reversed by venturicidin. Venturicidin was shown to act on the "stalk" part of the ATPase enzyme-complex, F_0 [23] and the results reported here are consistent with the mode of action of this inhibitor.

The rise time of the fluorescence decrease has not been resolved yet because of the low signal to noise ratio, but could be estimated to be lower than 100 ms (see Fig. 5). This rise time does not correspond to the decay time of the electro-chemical proton gradient (indicated for example by the carotenoid shift decay, see [23]) which is in the order of 2 – 4 sec. If the observed fluorescence decrease reflects only the conformational changes in F_1 which are essential for phosphorylation, one may conclude that the energy derived from the electro-chemical proton gradient is not exclusively used to induced those conformational changes which are energetically necessary to form ATP, as has been suggested by Boyer [9]. (In that case one would expect the rise of the conformational changes to be kinetically linked to the decay of the electrochemical proton gradient.) Further evidence is needed in order to clarify this problem, especially with respect to the origin of the fluorescence decrease of the labelled and re-attached coupling factors.

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