A Fluorescence Method for Measuring the Retention of Coupling Factor (CF_1) in Reconstitution Experiments of Photophosphorylation

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(Z. Naturforsch. **32 c**, 392–397 [1977]; received January 26/February 16, 1977)

Fluorescence Label, Coupling Factor 1, Reconstitution, Photophosphorylation

The recombination of chloroplast coupling factor 1 (CF_1) and thylakoid membranes in reconstitution experiments was studied through the fluorescence of paired labels covalently bound to NH_2-groups. It was found that maximum recombination is achieved at a ratio of 1.5–3 μg CF_1/μg chlorophyll. The addition of chloroplast lipids to the medium enhances the incorporation of CF_1 into the membranes.

The rates of ATP formation of the regenerated but labeled system are decreased to 50% of those found in unlabeled control experiments. This is discussed in context with the previous observation, that labeling of CF_1 at similar concentrations inhibits the ATPase activity of the isolated protein completely.

The possible use of double labeling in the study of the physical aspects of the reconstitution of the photophosphorylating system is discussed.

Introduction

Important aspects of the mechanism of energy coupling in photophosphorylation can be studied by reconstitution experiments. In these, the coupling factor (CF_1) is removed from the thylakoid membrane, thereby inactivating ATP formation, and subsequently added back to regenerate the system. The reconstitution attempts are generally successful, if thylakoid membranes are used which have not lost more than 30–50% of the native coupling factor by either EDTA treatment or pyrophosphate washing [1–3]. Such pretreated plastids lack, however, the capacity to maintain the high energy state and they show enhanced electron transport, two characteristics of uncoupling. It has been suggested that upon reconstitution of the photophosphorylating system, the renewed ability for ATP formation is solely provided by the previously present CF_1 [4].

The reconstitution experiment itself has two aspects. One is the purely physical problem of retention and integration of the added CF_1 into the thylakoid membrane. This structural aspect includes the parameters of interaction, *e. g.* binding sites, co-factors of binding, the role of Mg^{2+}, the specificity of CF_1 for combination with the thylakoid surface etc. The second concerns the mechanism by which the regenerated phosphorylating activity is maintained, *i. e.*, the functional aspect. An understanding of the latter is difficult without due regard to the former.

This paper concerns itself with a method which might give some information about the structural parameters involved in reconstitution experiments. It is based on a measurement of light energy transfer between fluorescent dye pairs, one attached to CF_1, the other to the thylakoid membranes. Experiments in this direction have been reported earlier by Kraayenhof and Slater [5] using only fluorescamine to label covalently CF_1 and De Kouchkovsky [6] who coupled chloroplasts with fluorescein-isothiocyanate (FITC).

The concept of our experiments is simple. CF_1 and chloroplasts are coupled with different chromophores, whose optical properties are chosen in such a way, that the emission band of the first overlaps with the absorption band of the second. Transfer of excitation energy between the two chromophores indicates a close proximity of the labeled proteins. In this way it is possible to measure the retention parameters of CF_1 in reconstitution experiments even in the absence of functional capability for photophosphorylation. These dye pairs used in our experiments were fluorescamine/fluorescein and fluorescein/rhodamine.

Abbreviations: CF_1, chloroplast coupling factor 1; DGD, digalactosyldiglyceride; FITC, fluorescein-isothiocyanate; MGD, monogalactosyldiglyceride; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; RITC, rhodamine-isothiocyanate; SL, sulfoquinovosyldiglyceride.

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This paper attempts an evaluation of the potential and shortcomings of this double labeling method.

Material and Methods

CF_1 was isolated from spinach as described by Lien and Racker[7]. In some instances the method of Strotmann *et al.*[8] was used to obtain relatively pure preparations of CF_1.

Chloroplasts, uncoupled but still retaining roughly 70 – 75% of their native CF_1, were prepared according to Schopf *et al.*[9] by washing in 0.5 mM pyrophosphate or according to Strotmann *et al.*[8] by treatment with EDTA.

The reconstitution procedure of Strotmann *et al.*[8] was used with only slight modification. Surplus, non reacted CF_1 was removed by double washing of the plastids. The control experiments contained instead of CF_1 solution the same amount of buffer.

Emission spectra were measured using the non commercial fluorimeter described by Harnischfeger[10]. The assay contained in 3 ml suspension chloroplasts equivalent to 10 – 30 μg chlorophyll.

Photophosphorylation was measured in the PMS system either through incorporation of ^{32}P or by the phosphomolybdat complex in the usual way (Schopf[11]).

Red saturating light ($\lambda > 600$ nm) was used in the measurements of the Hill reaction and of the proton gradient.

The dyes fluorescamine[12], fluorescein- and rhodamine isothiocyanate[13] were used as covalent labels for both chloroplasts and CF_1. Their absorption and emission bands overlap in the sequence fluorescamine (400 – 450 → 470 – 490), fluorescein (480 – 490 → 520 – 540), rhodamine (530 – 550 → 565 – 580). The shift in the maxima of absorption and fluorescence caused by covalent binding to proteins is relative small (± 15 nm) and can be neglected in these experiments.

The covalent coupling of marker and protein presented no difficulties in the case of fluorescamine. The procedure of Udenfriend *et al.*[12] could be followed completely. On the other hand, for the reaction between the fluorescent isothiocyanates and protein Goldmann[13] recommends long incubation periods (6 – 12 h at 0 °C, and high pH 9.5). Since this diminishes or even abolishes activity, the procedure was modified to an incubation of 45 min at pH 8, compensating for the reduced reaction time by an increase of dye concentration to 50 μM. A sufficient though not complete labeling was thus accomplished. Unreacted marker was removed from chloroplasts by repeated washing, from CF_1 by gelfiltration through Sephadex G-25.

The electrophoretic behaviour of CF_1 was not influenced significantly by the covalent label, though its ability for activation by heat was markedly decreased, probably by side reactions between marker and DTT. For obvious reasons an activation by trypsin treatment was impossible to accomplish in tagged protein.

Results and Discussion

The result of a reconstitution experiment in which EDTA treated thylakoids were labeled with FITC while CF_1 was marked with RITC is shown in Fig. 1. After recombination a marked increase in

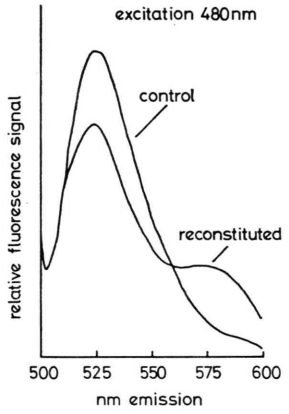

Fig. 1. Comparison of the emission spectrum of FITC labeled chloroplast membranes with that of the same preparation after reconstitution with RITC labeled CF_1.

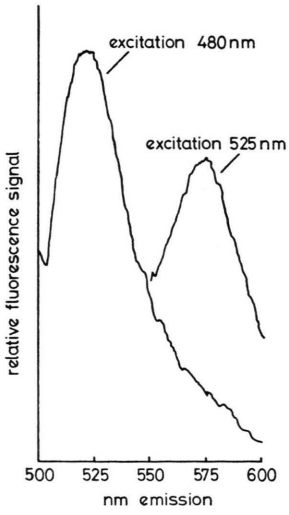

Fig. 2. Fluorescence emission spectra of a mixture of chloroplast membranes which were either labeled with FITC or RITC.

rhodamine fluorescence around 575 nm is observed when excited at 480 nm, while the emission of fluorescein, bound covalently to the chloroplasts, decreases.

In such experiments secondary effects have to be excluded. They can originate either in a slight absorption of RITC at 480 nm, or through the trivial effect, that energy emitted by fluorescein is reabsorbed by rhodamine. Such effects, however, can be neglected (Fig. 2). In this particular experiment a mixture of plastids was used, part of them labeled with RITC, the other half with FITC. The spectra can be resolved separately and do not show the secondary effects mentioned above.

The result in Fig. 1, thus, can be interpreted as a tight retention of CF_1 by the chloroplast membranes enabling energy transfer to occur. Such transfer is only possible if a minimum physical distance between the covalently bound pigments is achieved. It follows that the CF_1 has been incorporated into or attached itself tightly to the thylakoid membrane.

This explanation is strengthened by the fact, that several washes of the reconstituted system in reconstitution medium without CF_1 did not alter the energy transfer characteristics.

The validity of the conclusion is illustrated in Fig. 3, where a different combination of label was used. In this experiment CF_1 was labeled with fluorescamine while the thylakoid membrane carried covalently bound fluorescein. Excitation was at 365 nm, the absorption region of fluorescamine. The retention of the protein after reconstitution is clearly in evidence. The difference spectrum shows an emission optimum of fluorescamine at 465 nm. The additional emission at 512 – 515 nm, due to fluorescein, shows the energy transfer between fluorescamine and fluorescein and indicates, thus, a tight attachment of CF_1 to the membrane. The two emission bands between 400 and 450 nm are instrumental artefacts due to the cuvette and the assay medium. They provide a convenient internal standard for normalization.

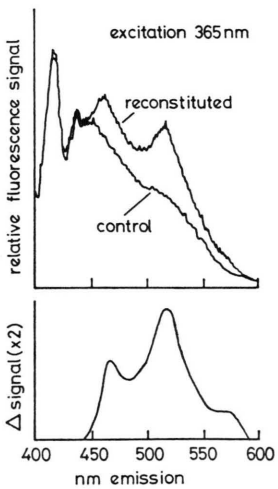

Fig. 3. Fluorescence emission spectra of FITC labeled thylakoid membranes before and after incubation with fluorescamine-labeled CF_1 in a reconstitution experiment. The resulting difference spectrum is shown in the lower half of the figure.

The interdependence of fluorescence emission and CF_1 retention is described in Fig. 4. As in all previous experiments surplus, non bound CF_1 was removed after reconstitution by several washings. If the fluorescence intensity is used as criterion for saturation, between 1.5 – 3 μg CF_1 protein/μg chlorophyll suffices. This was confirmed in a series of experiments although there are differences between EDTA and pyrophosphate prepared CF_1 depleted thylakoids.

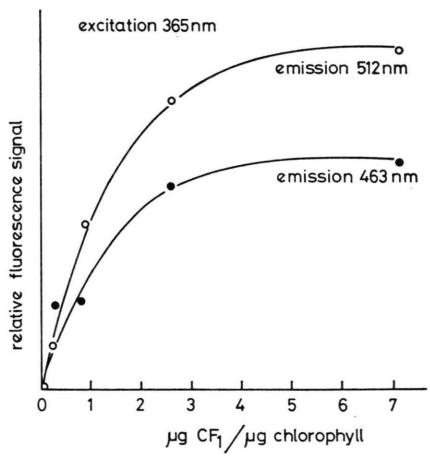

Fig. 4. Dependence of fluorescence emission of reconstituted thylakoids on the CF_1/Chl ratio in the reconstitution assay. Excitation 365 nm, CF_1 was labeled with fluorescamine (F_{463}), the thylakoids were labeled with FITC (F_{512}).

A more general problem concerns the nature of the binding sites and the capacity of thylakoids to bind CF_1 in relation to phosphorylation activity. Strictly quantitative informations are unlikely since both EDTA and pyrophosphate treatment of chloroplasts releases other membrane components besides CF_1. As shown previously by Heise and Jacobi [14] and Schopf *et al.* [9] a release (during pyrophosphate

and EDTA washings of plastids) and also a retention of lipids (during the reconstitution assay) takes place. To test their effect on the retention of CF_1, reconstitution experiments were performed in the presence of isolated chloroplast lipids. The CF_1 used had been purified previously with diisopropylether and hexane in order to remove all lipids. Table I shows that under these circumstances an increase in fluorescence emission is observed. Since CF_1 was labeled with fluorescamine and the thylakoids with FITC the increase indicates a better incorporation of CF_1 into the membrane and a consequently higher energy transfer between both markers. It seems at a maximum if the sulfolipid fraction is used which is in accordance with the results of Heise [15] and Schopf *et al.* [9].

Table I. Influence of lipids on the subsequently measured fluorescence emission of the labels in the recombined system. The lipids were added in an amount of 100 μg each to the reconstitution assay of 0.55 ml. Excitation of the twice washed and resuspended (in 0.4 M sucrose — 5 mM $MgCl_2$ — 0.1 M Hepes pH 8) reconstituted thylakoids at 365 nm; 15 μg Chl/measuring assay. The data have been normalized and the control arbitrarily set as 100. CF_1 labeled with fluorescamin (1.1 mg Prot/ml, 10 mM fluorescamine) thylakoids with FITC.

Addition	Relative signal F_{463}	Relative signal F_{517}	Ratio 517/463
—	100	100	1.0
DGD	258	238	.92
MGD	225	200	.89
PC+PI	276	223	.81
SL+PG	270	307	1.13

Direct information about the actual regeneration of phosphorylation activity, however, cannot be obtained through fluorescence measurements. The conclusions about spatial parameters obtained from the fluorescence experiments have always to be combined with functional data measured in the usual way.

To characterize the correlation between label and activity in more detail it has to be considered, that the label might influence the activity of the isolated components (CF_1 or thylakoids) in a different way than the recombined system.

No effect of the labels was found on the electron transport capacity of the chloroplast membranes used in the reconstitution experiments, which is in accordance with results reported by De Kouchkovsky [6] for FITC. It should be noted, however, that labeling of whole chloroplasts leads to a 50% inhibition in the uncoupled state while basal electron transport is unaffected [11].

The effect of the label fluorescamine on the ATPase activity of isolated CF_1 will be reported elsewhere [16]. Labeling of CF_1 by fluorescamine is complete at concentrations of $100-200$ μM. These amounts also resulted in a 90% inhibition of ATPase activity independent of the method of activation.

While the experiments of Schopf and Harnischfeger [16] argue for an almost complete deactivation of CF_1 by fluorecsamine, the activity of the reconstituted CF_1-thylakoid system is not affected this way.

Table II represents a comparison of the size of the proton gradient after reconstitution using unlabeled, partially CF_1 depleted thylakoids and CF_1, marked prior to the experiment with increasing amounts of fluorescamine. A reduction of the size of the proton gradient is seen only at fluorescamine concentrations higher than necessary to achieve maximum fluorescence in the isolated CF_1.

Table II. Influence of fluorescamine labeling of CF_1 on the functional regeneration of the proton gradient. The reconstitution assay contained in 1 ml 140 μg chlorophyll and 260 μg CF_1, labeling previously with fluorescamine (235 μg CF_1/ml labeling assay).

μM Fluorescamine in the labeling assay	Proton gradient (μmol H^+ · mg Chl^{-1})
0	299
50	318
100	321
250	276
500	184
Control no addition of CF_1	96

The result indicates, that any regeneration of phosphorylating activity might rest in the 75% CF_1 not removed by the previous pyrophosphate or EDTA treatment of the chloroplasts used in the experiment. Assumed in this deduction is, however, an equivalence of the mechanism of ATP-ase activity *in vitro* and ATP formation *in vivo*.

Fig. 5 shows the dependence of the regenerated phosphorylation activity on the CF_1/Chl ratio of the reconstitution assay. Unmarked CF_1 was recombined with FITC-labeled or unlabeled, CF_1-depleted membranes. The labeled membranes show $30-50$% less regeneration of phosphorylation activity than the unlabeled, control thylakoids. The rate of 150 μmol · mg $Chl^{-1} \cdot h^{-1}$ is nevertheless considerable. The optimum CF_1/Chl ratio is again around 1.5.

Fig. 6 shows the influence of the fluorescamine-concentration used to label CF_1 for the subsequent reconstitution experiment with either FITC coupled or unlabeled thylakoids. The CF_1/Chl ratio in the assay was in the optimum range of 1.5. The data show, that the amount of fluorescamine label in CF_1 has no influence in the case of FITC-marked thylakoids. A decrease around 30% is observed however in the control using unlabeled thylakoids.

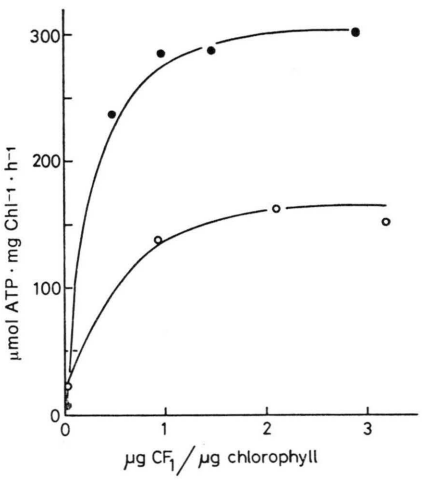

Fig. 5. Influence of the CF_1/Chl ratio during reconstitution on the functional regeneration of cyclic photophosphorylation. Only the thylakoid membranes were labeled with FITC. The control (—●—) and also CF_1 were unlabeled.

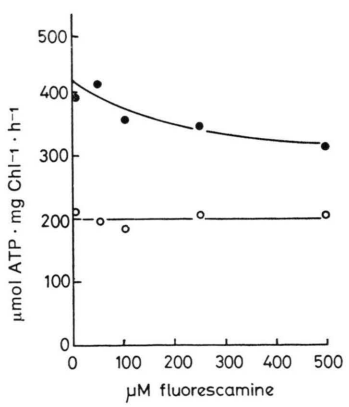

Fig. 6. Influence of CF_1 labeling with fluorescamine on subsequent regeneration of cyclic photophosphorylation. The thylakoid membranes of the reconstitution assay were either unlabeled (control —●—) or marked with FITC (—○—).

The experiment argues that the amount of covalent label carried by and located exclusively at the freshly attached CF_1 must in some way influence the entire system.

This observation is not easy to interpret as a general inhibition of energy coupling between electron transport and CF_1, especially since the labeled CF_1 is largely inactive [16] and the phosphorylation activity should rest in the residual factor carried by the thylakoid membrane. Experiments by Berzborn and Schröer [23] support the latter part of this argument.

An alternative explanation is the assumption of diminished mobility of CF_1 on or in the thylakoid membrane. Berzborn *et al.* [17] observed such a mobility which can be interpreted as a prerequisite for efficient phosphorylation. The observation that in Fig. 6 already a decrease in activity is observed if unlabeled CF_1 is attached to FITC labeled thylakoids, argues for this notion. It implies that the mobility and not alterations in CF_1 configuration [5] are the decisive factor under the experimental circumstances.

In summary, of the two parameters involved in an analysis of reconstitution the physical aspect seems to be well suited to fluorescence studies by the method of double labeling. Not only does the method allow direct measurements of a retention of CF_1 on or within the thylakoid membrane but it can provide also information about proximity of membrane and CF_1-aminogroups. Further development might include an evaluation of conformational changes occuring in the membrane bound coupling factor which have been demonstrated through other means [18–20]. These can be determined conceivably through changes in fluorescence polarization p which is dependent on the conformation of the labeled species [21, 22]. However, considerable theoretical work is necessary to develop the necessary equations applicable to such a complex system.

The technical assistance of Ms. S. Forbach is gratefully acknowledged. This study was supported by a grant from the Deutsche Forschungsgemeinschaft.

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