Studies on the Retention of CF_1 with or without Induced ATPase Activity by Pyrophosphate Treated Thylakoids and Its Relation to the Regeneration of Photophosphorylation

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The reconstitution of chloroplast coupling factor 1 (CF_1) into thylakoid membranes was investigated by the fluorescence of the covalently attached label fluorescamine. In contrast to a functional regeneration of ATP synthesis, a rebinding of CF_1 was observed regardless if the protein was in its native, purified state or had been activated for ATPase activity by heat, dithiothreitol (DTT) or trypsin treatment. The reintegration of CF_1 into the thylakoid membrane was estimated to be almost quantitative.

Since the label binds covalently and irreversibly to free NH_2-groups, its effects on the various functional parameters were investigated. Blocking of NH_2 groups leads to inhibition of ATPase activity in isolated CF_1. Labeled factor used in a reconstitution experiment on the other hand, does still result in considerable regeneration of ATP formation.

Attaching the label to whole thylakoids leads to a differential effect on phosphorylation, which is inhibited, and the H^+-uptake which seems largely unaffected. A differential effect is also observed on coupled and uncoupled electron transport.

The results are interpreted as evidence for an involvement of free NH_2-groups in the mechanism of photophosphorylation.

Introduction

The success of reconstituting phosphorylation activity by recombination of CF_1 and CF_1-depleted thylakoid membranes depends largely on the amount of proteins not extracted from the plastids during EDTA or pyrophosphate treatment (review by Nelson[1]). The critical lower limit for sufficient regeneration is a loss of more than 50% of native CF_1 during the preparation of CF_1 depleted membrane particles[2]. This observation has been attributed to an insufficient retention of the purified CF_1 due to changes in surface properties leading *e. g.* to aggregation. Alternative though related is the notion that its close to complete extraction through either EDTA of pyrophosphate treatment leads to irreparable destruction of membrane structure[2]. A third possibility derives from the argument that the protein retained during the reconstitution experiment serves only in a structural capacity (resealing of "holes") while the catalysis of ATP formation is restricted to the residual, native CF_1[3]. In this case the less residual CF_1, the less the maximum theoretical activity (on a chlorophyll basis) obtainable in a reconstitution experiment. It is possible that all three factors contribute to the aforementioned observation of a critical CF_1 concentration retained in the "CF_1-depleted" membranes.

One approach to investigate if retention of protein constitutes the critical factor is the use of fluorescent covalent labeling techniques which was employed by Kraayenhof and Slater[4] to study conformational phenomena of CF_1 activity. Similarly, a double fluorescent label was suggested by Harnischfeger and Schopf[5] to investigate the physical parameters of membrane-CF_1 interaction. CF_1 was labeled in both cases with fluorescamine which reacts specifically with exposed amino groups. Only the covalently bound dye possesses a strong fluorescence whereas the molecule itself and its hydrolyzation products are non-fluorescent.

This paper describes the influence of the fluorescamine label on the activity of isolated CF_1, the reconstitution of activity with labeled CF_1 and also the effects of ATPase-activation on labeled and unlabeled protein with regard to reconstitution. The experiments suggest that retention of protein during reconstitution is not the critical factor limiting ac-

tivity for a regeneration of photophosphorylation. In addition applying the label to the thylakoids rather then to CF_1 suggests, that in the mechanism of phosphorylation and electron transport free amino groups play an important role.

Material and Methods

Coupling factor CF_1 was prepared according to Lien and Racker[6] with the modification that the chloroplasts were washed prior to EDTA treatment with 5 mM pyrophosphate at pH 7.4 in order to remove stroma proteins and carboxydismutase[7]. The protein, pure according to electrophoretic observation, was stored in 20 mM $Tris - SO_4 - 2$ mM $ATP - 2$ mM EDTA at pH 7.1.

For the use in the experiments the CF_1 preparation was centrifuged, the precipitate resolved in 10 mM Tricine pH 8 and passed through a Sephadex G-25 column $(20 \times 1.5$ cm). After elution with 20 mM Tricine pH 8.0 the CF_1 was effectively separated from $(NH_4)_2SO_4$ as determined by the Neßler test.

ATPase activity was determined through released inorganic phosphate using the method of Taussky and Shorr[8]. Activity of ATPase was induced through either trypsin incubation[9], DTT treatment[10] or heat exposure[11].

CF_1 was labeled with fluorescamine in the concentrations given in the legends. Since the dye is insoluble in water, it was added in acetone solution. The amount of acetone in the labeling assay did not exceed 5%. The labeling assay contained bicarbonate buffer (10 mM, pH 8.3)in order to prevent any possible influence through the amino groups of Tricine. Fluorescence was measured in the fluorimeter described by Harnischfeger[12], excitation was at 365 nm, the emission spectrum was recorded between 400 and 500 nm.

Uncoupled thylakoids which have lost between $25 - 30\%$ of their native CF_1 were prepared according to Schopf et al.[13]. They were partially depleted of CF_1 by hypotonic treatment with 0.5 mM pyrophosphate of pH 7.4. Chlorophyll concentration at this step was 0.1 mg/ml.

Cyclic photophosphorylation was assessed in the PMS system. The assay contained in 2.2 ml: 100 μmol Tricine pH 8, 10 μmol $MgCl_2$, 5 μmol ATP, 10 μmol Na_2HPO_4 including ^{32}P, 100 μmol PMS and 40 μg chlorophyll eq. chloroplasts. Illumination was with 9.5×10^5 erg/$cm^2 \times$ sec of light with a wavelength of >610 nm. The determination of $AT^{32}P$ formed was according to Lindberg and

Ernster[14] using Cerenkov emission in the liq. szintillation assay[15].

The fluorescence of 9-aminoacridine, used as an indication of membrane energetization, was measured as described before[12]. The determination of the size of the proton uptake was accomplished with a sensitive glass electrode (Ingold LOT 405-M5) attached to a Metrohm pH meter. Starting pH of the assay was 6.2, illumination by red light $(\lambda > 610$ nm) of an intensity of 9.5×10^5 erg $\times$ cm^{-1} $\times sec^{-1}$. The assay contents were similar to those described by Strotmann[16].

In the reconstitution assay the uncoupled thylakoids and purified CF_1 were recombined. The medium contained in addition 0.1 M sucrose, 25 mM Tricine pH 8, and 10 mM $MgCl_2$. The reconstituted system was assayed for photochemical activity after an incubation time of 20 min at 0 °C.

Results

1. The retention of CF_1 activated for ATPase activity

Our experiments confirm the initial observation by Jagendorf[17] that a regeneration of photophosphorylation is impossible with CF_1, in which ATPase activity was induced by treatment with trypsin or heat. It can only be achieved, if the activation was obtained by DTT exposure (Table I).

Table I. Regeneration of cyclic photophosphorylation using CF_1 in which ATPase activity was induced prior to the reconstitution experiment.

Thylakoids	CF_1	Phosphorylation activity	ATPase activity of CF_1
Control	—	419	—
P.P. washed	—	4	—
P.P. washed	$+CF_1$	123	—
P.P. washed	$+DTT-CF_1$	129	5.9
P.P. washed	$+trypsin-CF_1$	3	6.4
P.P. washed	$+heat-CF_1$	10	6.2

Activity of phosphorylation in the PMS system given in μmol ATP formed$\times$mg $Chl^{-1} h^{-1}$; rate of ATP hydrolysis by activated CF_1 (type of activation indicated by prescript) given in μmol ATP $\times$ mg $protein^{-1} min^{-1}$. DTT-CF_1 was purified before reconstitution by Sephadex G-25, the removal of DTT did not influence ATPase activity.

Further investigation showed, that the reconstituting capacity is already lost long before the trypsin treatment yields the maximum rate of ATP hydrolysis (Fig. 1). In the same manner, the 9-AAC
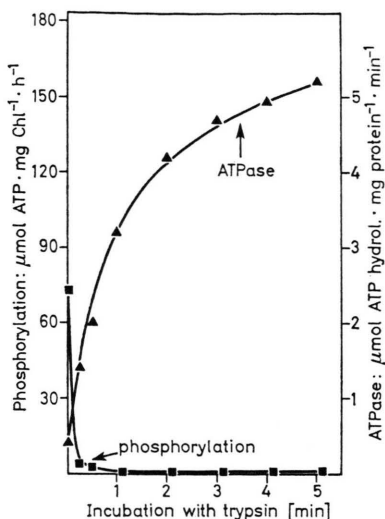
Fig. 1. Influence of trypsin treatment of CF$_1$ on its ability to regenerate phosphorylation in a reconstitution experiment. The rate of ATP formation in the untreated control was 290 μmol $\times$ mg Chl^{-1} h^{-1}.
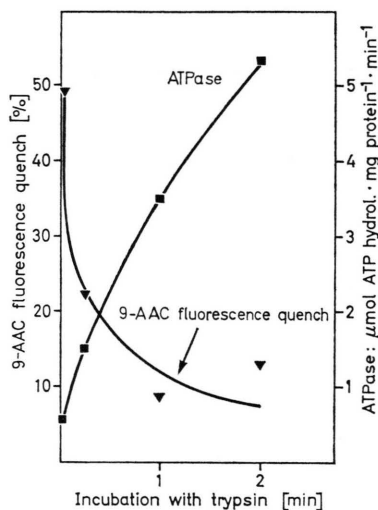

Fig. 2. Influence of trypsin treatment of CF$_1$ on its ability to regenerate the 9-AAC quench after reconstitution.

Fig. 3. Concentration dependence of CF$_1$ retention of thylakoids. CF$_1$ (0.9 mg/ml) was labeled with 250 μM fluorescamine and used in increasing amounts for this series of reconstitution experiments. Fluorescence was measured after two washes of the reconstitution assay to remove excess CF$_1$.

tion of the reconstitution mixture unreacted CF$_1$ can be removed by centrifugation and washing, and the amount of CF$_1$ retention can be estimated by the remaining fluorescence signal. (Two washes of the reconstituted particles in the incubation medium sufficed in our experiments to remove all fluorescence emission due to the fluorescamine-protein adduct in the supernatant.) Fig. 3 shows the result of such an experiment with purified, labeled, coupling factor. Approximately 3 – 4 mg CF$_1$/mg Chl sufficed to saturate retention. The actual amount of coupling factor incorporated into the membrane is quite small, only 3% of the added protein under our conditions. This value was calculated in Table II from the relative size of the fluorescence signal and estimated to be around 0.1 mg protein/mg chlorophyll. This corresponds to the amount of CF$_1$ released during pyrophosphate washing [13].

Table II. Determination of the amount of CF$_1$ rebound to thylakoid membranes during reconstitution.

	Relative fluorescence signal eq. μg CF$_1$ ($F_{365 \rightarrow 470}$)	
Entire reconstitution assay	820	655
Reconstituted thylakoids after removal of unreacted CF$_1$	17	13.6

CF$_1$ (0.9 mg/ml) was marked with 250 μM fluorescamine prior to use in the reconstitution assay which contained besides the above amount of CF$_1$, 136 μg chlorophyll eq. uncoupled thylakoids. The fluorescence signal was determined before and after removal of non reacted CF$_1$ using aliquot samples.

quench is diminished after reconstitution with the briefly activated coupling factor (Fig. 2).

These observations lead to the question whether the ATPase-activated CF$_1$ does not bind to the thylakoid or whether it is only inactive but fully integrated into the membrane. One possible solution is offered by reconstitution experiments with CF$_1$ to which a fluorescent label was attached through exposure to fluorescamine. After a sufficient incuba-
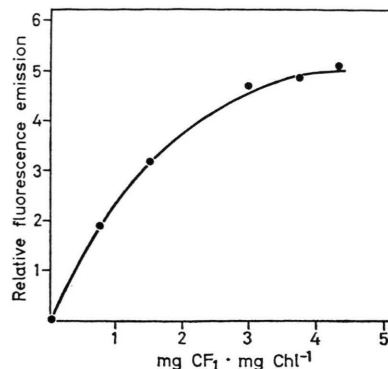
The coupling of fluorescamine with purified CF₁ is influenced by a number of factors which can distort the information obtainable from reconstitution experiments. One poignant example is given in the model reaction of Fig. 4 where lysine labeled with fluorescamine was used. The presence of DTT or ATP during labeling reduces the emission of the adduct. ATP is itself susceptible to the label and can, therefore, influence the result. The DTT dependent decrease was, however, not observed when the compound was added after the labeling reaction. DTT must, therefore, interfere with the chemical mechanism forming the covalent bond.
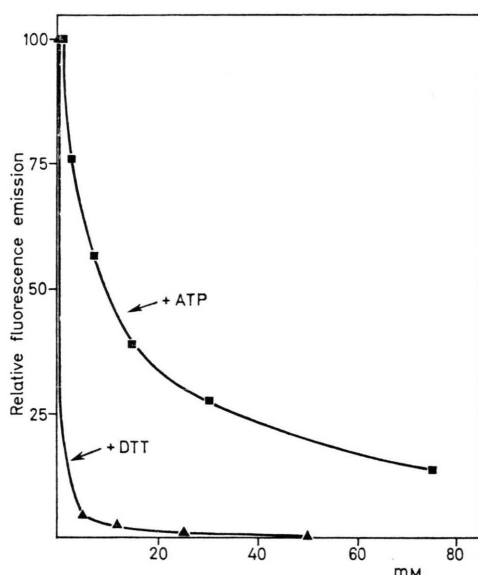

Fig. 4. The influence of DTT and ATP on the fluorescence signal of the fluorescamine adduct. The label (250 μM) was attached to lysine (10 mM). The two components were present in the conc. given during the labeling procedure.

This also can explain the observation of Kraayenhof and Slater[4], that fluorescamine labeling of CF₁ is much diminished after DTT treatment. Their interpretation, that amino groups exposed in the native enzyme are buried after ATP-ase activation with DTT is, therefore, open to question. Consequently, in order to measure the retention of the ATPases, it was necessary to separate prior to reconstitution those substances which were added for the activation of CF₁.

In the case of DTT, the activated protein was separated by column chromatography with Sephadex G-25 and subsequently labeled with fluorescamine.

Taking this precaution the retention of activated CF₁ in reconstitution experiments is almost identical regardless of the procedure used to induce ATPase activity. Moreover, as summarized in Table III, the fluorescence signal of the rebound protein was comparable to that of the uninduced coupling factor. The retention of the trypsin treated protein did not change even after 5 min of trypsin exposure. This finding does not agree with experiments by Racker et al.[18] who observed a diminished degree of retention of trypsin activated CF₁-ATPase, measured by ATP hydrolysis of the supernatant after reconstitution.

Table III. Influence of ATPase induction in CF₁ on its rebinding capacity in reconstitution experiments.

	Rel. fluorescence signal ($F_{365 \to 470}$)
CF₁, purified	10.4
DTT-CF₁	10.6
Heat-CF₁	9.8
Trypsin-CF₁	10.8

Incubation of CF₁ for full activation of ATPase activity: DTT — 3 h, trypsin — 1 min, heat (64°) — 4 min. Labeling with 250 μM fluorescamine after removal of byproducts and activation reagents. Reconstitution and other procedures as described in methods and in the legend to Table II.

2. The reconstitution of the proton uptake and photophosphorylation with labeled CF₁

Although the retention of CF₁ in the reconstitution experiment is complete as shown in Table II, the capacity for energy conservation of the reconstituted system should nevertheless be influenced by the label of the coupling factor.

We observed, that the fluorescamine label affects markedly the ATPase activity of isolated CF₁. Fig. 5 shows that the hydrolytic capacity is inhibited around 90% at the concentration of 100 μM fluorescamine in the labeling assay. If the fluorescence emission of the marked CF₁ serves as indicator, this concentration corresponds to a saturation of the exposed amino-groups (Fig. 6). Since trypsin by its proteolytic action generates new amino terminal groups, more fluorescamine can react with them and the respective curve in Fig. 6 is shifted towards higher concentrations. These results indicate, that the fluorescamine labeled CF₁ used in the reconstitution experiments is enzymatically inactive, pro-
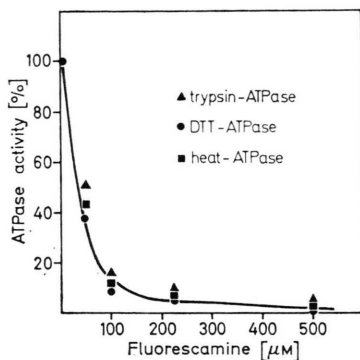
Fig. 5. Influence of the fluorescamine label on the ATPase activity of isolated CF_1. The protein (0.2 mg/ml) was labeled after activation by the methods indicated. Activity of 100% is equal to 13.4 μmol ATP hydrolysed $\times$ mg prot.$^{-1}$ min^{-1} for trypsin activated CF_1, 5.6 for heat activated and 6.1 for DTT activated protein.
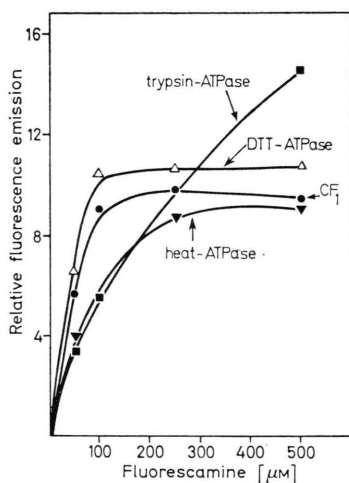

Fig. 6. Fluorescence saturation curve for fluorescamine labeling for isolated CF_1. The protein (0.2 mg/ml) was labeled after activation as indicated.

vided, phosphorylation and hydrolysis of ATP proceed by a similar mechanism involving the same catalytic sites.

However, as demonstrated in Fig. 7, the proton uptake and the phosphorylation is similarly reconstituted if unlabeled CF_1 or a coupling factor which was marked with fluorescamine was used. Only higher concentrations of label inhibit slightly. At 250 μM fluorescamine, which was generally used in the experiments described before, the reconstitution is almost the same as with the unlabeled coupling factor. The absolute values for the controls were 138 μmol of ATP $\times$ mg Chl^{-1} h^{-1} and 168 nmol H$^+$ $\times$ mg^{-1} chlorophyll for the proton uptake corresponding to a 30% regeneration.
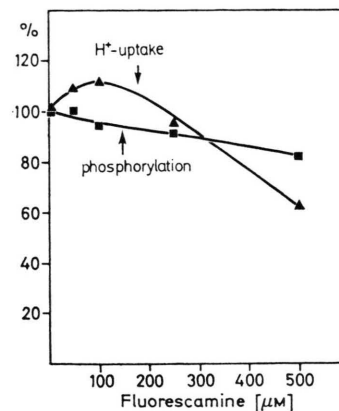

Fig. 7. Influence of fluorescamine labeling of CF_1 on its ability to regenerate the proton uptake and phosphorylation in reconstitution experiments. CF_1 (0.9 mg/ml) was labeled with the amount of fluorescamine indicated prior to the reconstitution assay. 100% activity were 138 μmol ATP $\times$ mg Chl^{-1} h^{-1} (7 μmol before reconstitution) and 168 nmol H$^+$/mg Chl (16 nmol before reconstitution).

3. Reconstitution with labeled thylakoids

The free amino groups seem to play an important role in the coupling mechanism of phosphorylation. This is evident from the experiment shown in Fig. 8, where control type thylakoids (shocked but still possessing their full complement of CF_1) were exposed to various concentrations of fluorescamine. A differential effect on photophosphorylation and proton uptake is observed. At 250 μM the former was inhibited, the latter still unaffected. Basis electron transport (FeCy acceptor) is not influenced by the label, while the uncoupled activity is pronouncedly decreased (Fig. 9).
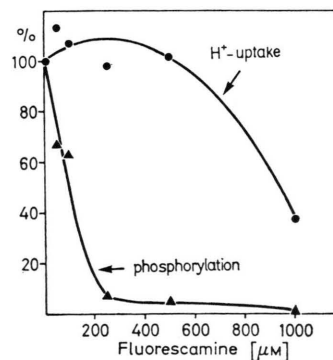

Fig. 8. Influence of fluorescamine labeling on phosphorylation and proton uptake of intact thylakoids. The thylakoids (0.2 mg Chl/ml) were labeled with the fluorescamine concentration indicated. The 100% values are 147 nmol H$^+$·mg Chl^{-1} and 121 μmol ATP·mg Chl^{-1}·h^{-1} for photophosphorylation (FeCy-acceptor).
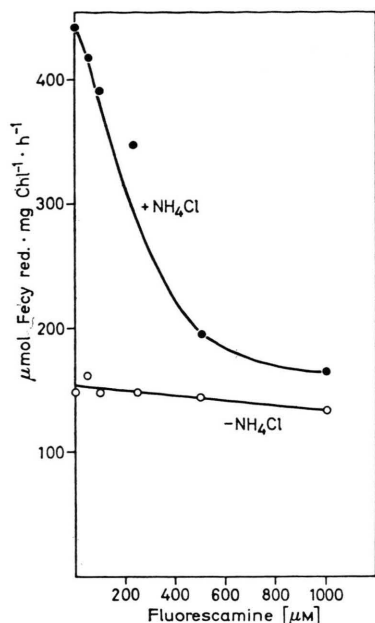
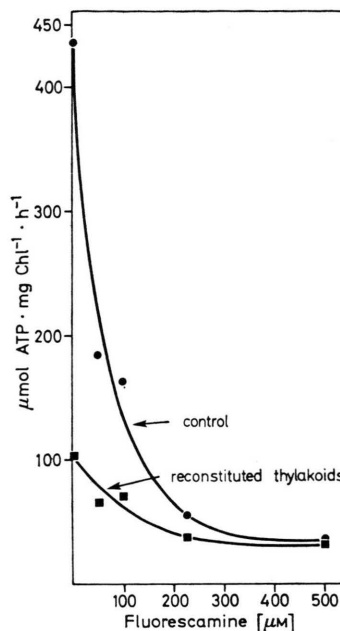Fig. 9. Influence of fluorescamine labeling on electron transport of intakt thylakoids (FeCy-acceptor).

Fig. 10. Influence of thylakoid-membrane labeling by fluorescamine on the ability to regenerate cyclic photophosphorylation in reconstitution experiments. Chlorophyll concentration in the labeling assay was 0.2 mg/ml.

Fig. 10 describes the effect of labeling thylakoids with fluorescamine on the regeneration of activity in a reconstitution experiment. In analogy to the above results an inhibition of cyclic phosphorylation is observed in both controls and reconstituted thylakoids leading to the same final value, though on a relative basis the influence on the reconstituted system is much less. This finding argues, that in these experiments the active (coupling?) site of the residual CF$_1$, which is not remvoed by washing from the uncoupled thylakoids, is affected, since labeled CF$_1$, on the other hand, does not greatly impair the reconstituted activity (compare Fig. 6).

Discussion

The experiments give information about both the structural and the functional aspect of reconstitution. From a structural point of view the finding is important, that all types of CF$_1$, normal or activated by the various means available, bind to the thylakoid membrane. A rough quantitative estimate from the fluorescence data gives a retention of 0.1 mg protein/mg Chl. The total amount of CF$_1$ in intact thylakoids has been given as $0.42 - 0.45$ mg CF$_1$/mg Chl[7, 19]. Taking this value into con-

sideration the amount rebound would correspond to about 30% of the total, i. e. exactly the protein removed through EDTA or 0.5 mM pyrophosphate treatment. The structural reconstitution thus seems quantitative.

Of special interest is the observation that CF$_1$ with trypsin induced ATPase activity is reintegrated into the membrane during reconstitution. Deters et al.[20] reported that under these conditions CF$_1$ loses its δ subunit and concluded from the absence of function that the protein did not rebind. Similarly Nelson and Karny[21] implied a strong correlation between binding of CF$_1$ and the δ subunit. If we imply, — though we have not shown it —, that in analogy to Deters et al.[20] trypsin treatment in our case also removes the δ subunit from CF$_1$, we have to conclude that the δ subunit cannot be the only structural link between CF$_1$ and thylakoid membrane.

The functional aspect, i. e. the ability to regenerate photophosphorylation, is more complex. Hesse et al.[2] imply 3 successive degrees of membrane breakdown during CF$_1$ extraction, namely, disorganization of grana stacking — removal of CF$_1$ — membrane perforation. They report membrane perforation as starting when about 50% of native CF$_1$ is ex-

tracted. Since the uncoupled thylakoids used in our experiments contain still about 70% of their native CF$_1$, holes in the membrane (not to be confused with the normal ion transmitting pores) and thus a resealing through the offered protein during reconstitution is not the decisive factor for regeneration of phosphorylation activity. As discussed by Jagendorf [17] the functional aspect of those CF$_1$ molecules that rebind is not clear. The regeneration of phosphorylation with inactive, fluorescamine labeled CF$_1$ implies, that the renewed activity rests mostly, but not necessarily exclusively, in the residual membrane factor (cp. also Berzborn and Schröer [22]). The result suggests that in this case the rebound CF$_1$ restores only membrane integrity which is required for energy conservation.

The labeling experiments seem to indicate also that free amino-groups play a role in the coupling between phosphorylation and electron transport. These groups are virtually abolished through the covalent attachment of fluorescamine [23] in saturating amounts. Although the proton uptake is unaffected at 100 μM fluorescamine, the phosphorylation is only 10% its original size. Thus, the energy reservoir, if proton uptake serves as a rough indicator, is still present but cannot be diverted into ATP formation. Labeling the thylakoids before reconstitution is much more sensitive than labeling CF$_1$, which however can be easily explained by a strictly structural organizational role of the rebound CF$_1$. NH$_2$-groups must be involved at the coupling site proper, since fluorescamine labeling decreases uncoupled electron transport in class I chloroplasts but leaves basal rates practically unaffected (Fig. 9).

The results so far indicate that although in reconstitution experiments CF$_1$ is retained at all the binding sites, the crucial feature is the repair of energy transfer between membrane and coupling factor. This problem warrants further investigation.

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