The Degree of CF_1 Release and the Reconstituting Capacity of the Depleted Membranes *

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(Z. Naturforsch. **32 c**, 600–604 [1977]; received April 29, 1977)

Pyrophosphate, Release of Coupling Factor, Reconstitution of Photophosphorylation

Uncoupling of photophosphorylation was shown in chloroplasts from spinach which were isolated in media containing pyrophosphate and thereafter suspended in salt-free solutions. The effective concentration of pyrophosphate to uncouple was determined by the addition of pyrophosphate to free thylakoid systems and shown to be dependent on the presence of the completely dissociated species which favoured complex formation with bivalent cations. Higher concentrations of pyrophosphate led to a stabilization of photophosphorylation. The uncoupling is due to the release of the coupling factor. The degree of liberation was shown to be dependent on the chlorophyll concentration. The capacity of extracted membranes to reconstitute photophosphorylation with their corresponding supernatant or with purified CF_1 was directly correlated to the amount of the liberated coupling factor.

The uncoupling of photophosphorylation in EDTA-treated chloroplasts [1, 2] was shown to be due to the release of the coupling factor CF_1 [3–6]. However, as reported by McCarty and Racker [4], the liberation of CF_1 was dependent on the concentration of EDTA used during the incubation. The release of CF_1 was found to be maximal at 10^{-3} M EDTA whereas higher concentrations failed to remove the protein from the surface of the thylakoid membranes.

Similar results are expected when other substances are used which have the same property to form complexes with bivalent cations. Among the various substances in use, pyrophosphate is of special interest. The isolation of intact chloroplasts is essentially improved when the isolation media contain pyrophosphate at higher concentrations [2, 7–9]. However, the photophosphorylation was shown to be completely abolished when this material was suspended in salt-free solutions under slightly alkaline conditions [10, 11]. The phosphorylating capacity was retained when the chloroplasts were suspended in solution of low ionic strength at pH 6 or in the presence of Mg^{2+}. Since the degree of uncoupling was shown to be accompanied by an increasing release of certain lipids, Heise and Jacobi suggested that in addition to an impaired membrane integrity the coupling factor was liberated by this treatment [10].

The present paper describes the extraction of thylakoids by pyrophosphate under various conditions. The capacity to reconstitute photophosphorylation was found to be related to the portion of CF_1 which remained on the membranes.

Material and Methods

Chloroplasts from spinach were isolated according to Jacobi [2] but without pyrophosphate. For the preparation of thylakoid systems, once washed chloroplasts were suspended in 1 mM Hepes buffer pH 7.4 with variable amounts of pyrophosphate as noted in the legends. After 20 min, the extracted membranes were centrifuged at 25 000 × *g* at 0 °C and resuspended in 0.1 M sucrose, 0.1 mM $MgCl_2$, 33 mM NaCl, and 10 mM tricine buffer pH 8.0. For the reconstitution, the corresponding supernatant or purified CF_1 was added to the extracted thylakoids in the presence of 10 mM $MgCl_2$ as described previously [3–6]. The reconstituted systems were concentrated by centrifugation if necessary. The rate of electron transport was followed by the reduction of ferricyanide. In some experiments, the rate was directly recorded as described before [11] in a special spectrophotometer equipped with attachements for cross illumination [12]. In the case of the coupled electron transport, the reactants and

* This paper is dedicated to the memory of Prof. Dr. G. Jacobi who initiated and encouraged many aspects of this work.

Abbreviations: P-P, inorganic pyrophosphate; P_i, inorganic orthophosphate; ADP and ATP, adenosine-di- or -triphosphate; PMS, phenazoniummethosulfate; 9-AAC, 9-Aminoacridine; tricine, N-[tris(hydroxymethyl)methyl]-glycine.

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thylakoids were pipetted into small Erlenmeyer flasks and illuminated for 3 min in a Warburg apparatus. Noncyclic phosphorylation was determined enzymatically via hexokinase and glucose-6-phosphate-dehydrogenase. Cyclic phosphorylation was measured according to Jagendorf and Margulies [13] with PMS, using red actinic light (>610 nm; 9.5 $\times 10^5$ ergs $\times$ cm^{-2} $\times$ sec^{-1}). Radioactive AT^{32}P was determined according to Lindberg and Ernster [14]. The proton gradient was measured with a glas electrode (Ingold LOT 405-M5) and a sensitive pH meter (Metrohm E 515). The reaction mixture contained in 4 ml (μmol): sucrose 400, NaCl 130, PMS 0.1, MES (pH 6.0) 0.8, and chlorophyll 120 – 150 μg. The mixture was adjusted to pH 6.2 and illuminated with red actinic light having the same characteristics as used for cyclic phosphorylation. For the calculation of the recorded proton gradient, the steady state value was determined and correlated to the signal after addition of a known amount of HCl.

The measurement of the 9-AAC fluorescence quench was similar as described by Bakker-Grunwald and van Dam [15]. A detailed description of the apparatus is given by Harnischfeger [16]. The reaction mixture contained in 3 ml [μmol]: tricine 15, pH 8.0, KCl 300, $MgCl_2$ 15, pyocyanine 0.030, 9-AAC 0.012; chlorophyll 50 μg. Pyocyanine was prepared according to Jagendorf and Margulies [13].

The coupling factor was isolated after Lien and Racker [17] with the modification of Strotmann *et al.* [18]. After washing with 5 mM pyrophosphate, CF_1 was released by 1 mM EDTA pH 8.0. The supernatant obtained after centrifugation was adsorbed on DEAE-Sephadex A-50 which was equilibrated with 20 mM tricine pH 7.1, 1 mM ATP, 2 mM EDTA, and 80 mM $(NH_4)_2SO_4$. After washing with 100 and 120 mM ammonium sulfate, CF_1 was eluted with 280 mM $(NH_2)_2SO_4$. The coupling factor was precipitated after 50% saturation with ammonium sulfate and stored in 20 mM Tris-SO_4, pH 7.1, 2 mM ATP and 2 mM EDTA in the cold. Under these conditions the protein was stable for at least 4 months without losing activity. The preparation led to a pure protein as judged by gel electrophoresis. The activation of ATPase by trypsin, heat or treatment with dithiotreitol was carried out according to the conventional methods [17]. After activation, the Ca^{2+}-dependent ATPase was measured by determination the P_i liberated from ATP [19].

Chlorophyll was determined according to Arnon [20] and protein after Lowry *et al.* [21]. The biochemical substrates and the enzymes were purchased from Boehringer & Soehne, Mannheim. PMS was a product from Sigma Chem., St. Louis, USA, ^{32}P was obtained from Amersham-Buchler, Braunschweig. The other chemicals came from Merck, Darmstadt, and were of analytical grade.

Results and Discussion

1. Photochemical activities of thylakoids from chloroplasts isolated in PP-media

Photophosphorylation can be completely abolished in thylakoid membranes from chloroplasts which were isolated in media containing PP (Table I). These chloroplasts were isolated according to Heise and Jacobi [10] (0.4 M mannitol, 20 mM NaCl, 20 mM tricine, 10 mM PP, pH 7.4) and thereafter suspended in salt-free solutions at pH 8.9. In Fig. 1 is shown that the uncoupling was solely due to PP. With increasing amounts of PP in the isolation medium, the proton gradient decreased continously and was completely abolished at 5 mM PP. Moreover, the degree of uncoupling was directly correlated with the amount of liberated CF_1. But after isolation with 2.5 mM PP about 80% of CF_1 remained membrane bound whereas the proton gradient is nearly abolished.

In these experiments PP was only present in the isolation media but not during hypotonic treatment. The observed effect is thought to be due to that amount of PP which was taken up or adsorbed at the intact chloroplasts during the homogenization of leaves and the subsequent washing steps. Therefore, the actual concentration of PP which led to uncoupling during the hypotonic treatment is definitely lower.

Table I. The influence of pyrophosphate present in the media for the isolation on the photochemical activity of broken chloroplasts. Chloroplasts were isolated in 0.4 M mannitol containing 20 mM NaCl and 20 mM tricine pH 7.4 (medium I). The medium designated as II, contained in addition 10 mM PP. The once washed chloroplasts were broken in 5 mM glycylglycine pH 8.9 and diluted up to 0.1 mg chlorophyll/ml. The photochemical activities are expressed in μmol ATP formed $\times$ mg chlorophyll^{-1} $\times$ hour^{-1}, μmol ferricyanide reduced $\times$ mg chlorophyll^{-1} $\times$ hour^{-1} or nmol H^+ $\times$ mg chlorophyll^{-1}. The reaction mixtures for noncyclic phosphorylation contained in 3 ml (μmol): tricine (pH 8.0) 100, $MgCl_2$ 10, ferricyanide 4.5, P_i 10, ADP 5 and chlorophyll 60 – 80 μg. The proton gradient was measured in 4 ml reaction vessel containing (μmol): sucrose 400, NaCl 130, PMS 0.1, MES (pH 6.0) 0.8 and chlorophyll 120 – 150 μg.

Isolation medium	PP	Proton gradient	Phosphorylation	Electron transport	P/2e
I	–	237	131	284	0.92
II	+	5	0	457	–

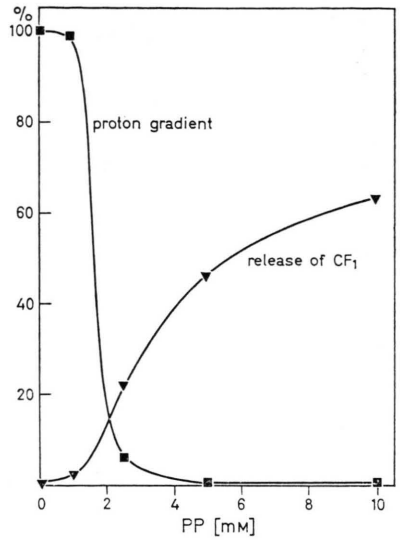

Fig. 1. The influence of pyrophosphate concentration in the isolation medium on the release of CF_1 in broken chloroplasts. Chloroplasts were isolated as indicated in the legend of Table I and suspended in 5 mM glycylglycine pH 8.9. The release of CF_1 was determined by the activity of trypsin-activated ATPase in the supernatant. 100% correspond to 342 μmol of hydrolyzed ATP × mg chlorophyll^{-1} × hour^{-1} or 203 nmol H^+ × mg chlorophyll^{-1}.

2. *Incubation of free thylakoid membranes with PP*

In order to determine the concentration of PP which led to uncoupling the chloroplasts were isolated in media without PP but subsequently suspended in hypotonic solutions with varying concentrations of PP. As shown in Fig. 2 the highest degree of uncoupling was observed after treatment with 0.5 mM PP at pH 7.4. Moreover, as demonstrated in Figs 3 and 4 there is a direct correlation between uncoupling and release of CF_1. Increasing concentrations of PP stabilize the thylakoid system in a similar manner as described for EDTA [4].

The effect of PP on the photophosphorylation is suggested to be due to the complex formation with bivalent cations similarly as reported for EDTA [3–6]. Since the completely dissociated molecule causes the binding of bivalent cations the pH during the incubation is similarly important as the total concentration. The values for pK_4 listed in the literature vary largely between pH 8.2 and 9.6 (Good [22], Umbreit *et al.* [23], Long [24], D'Ans-Lax [25]). If one assumes a pK_4 of 8.75, the degree of dissociation is less than 0.1% at pH 5.5 and approximately 60% at pH 8.9. Consequently, the uncoupling at pH 8.9 and the stabilization at pH 5.5 as described recently [10] are simply explained by the different degree of dissociation.

The effect of pH during the incubation with PP on the photochemical activities and on the release of CF_1 is summarized in Fig. 2. With increasing pH the rate of ATP-formation decreased continu-

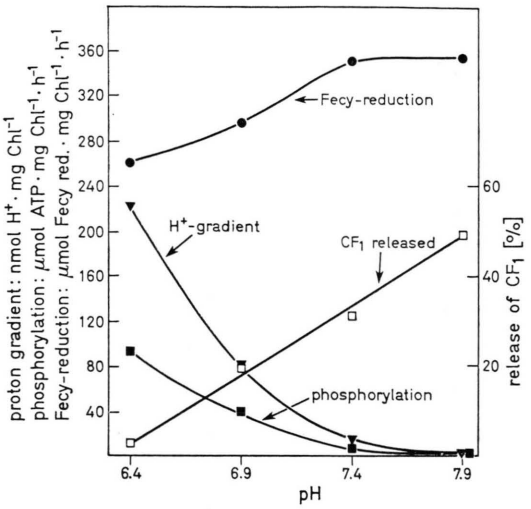

Fig. 2. The influence of pyrophosphate at different pH on the photochemical activities of thylakoids. Chloroplasts were suspended in 5×10^{-4} M PP at pH as indicated. 100% CF_1 correspond to an activity of 384 μmol of hydrolyzed ATP × mg chlorophyll^{-1} × hour^{-1}. Ferricyanide reduction and ATP-formation are expressed in μmol × mg chlorophyll^{-1} × hour^{-1}. Proton gradient in nmol H^+ × mg chlorophyll^{-1}.

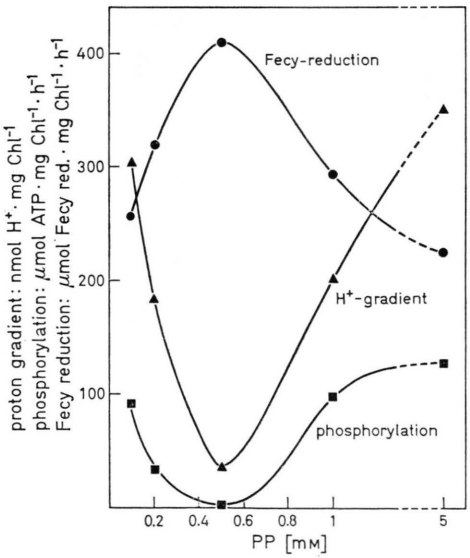

Fig. 3. The influence of the pyrophosphate concentration on the photochemical activities of thylakoids. The chloroplast pellet was suspended in PP as indicated at pH 7.4. Final chlorophyll concentration 0.1 mg/ml. The activities are expressed as noticed in the legend of Fig. 2.

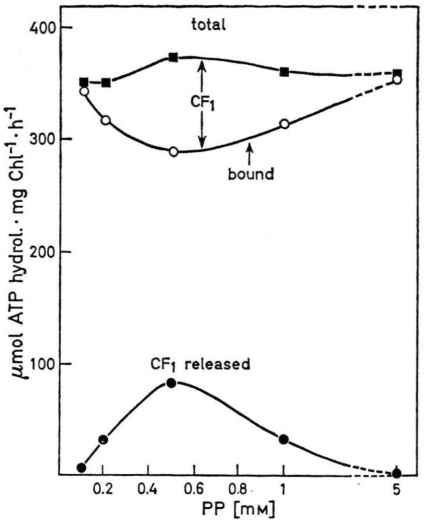

Fig. 4. The influence of pyrophosphate concentration on the release of CF_1. The thylakoids were suspended in PP of pH 7.4.

ously while the electron transport was progressively enhanced. This tendency was correlated with the liberation of the coupling factor. Whereas only 2 – 3% of CF_1 were released at pH 6.4, approximately 50% were found to be liberated at pH 7.9. It is of interest to note that membranes which were prepared by incubation with PP at pH 7.4, were completely uncoupled but retained approximately 70% of their coupling factor.

3. *The reconstitution of photophosphorylation*

The membranes which were extracted with 5×10^{-4} M PP at pH 7.4 were used as standard material for the reconstitution. Similarly as in the case for EDTA extracted thylakoids [6], the capacity to reconstitute the photophosphorylation with CF_1 was strongly dependent upon the chlorophyll concentration during the incubation with the chelator. In addition, as shown in Table II, the impaired regeneration of ATP-formation with increasing dilution was associated with a higher degree of CF_1 release. However, a high extent of reconstitution was only obtained in those membranes which retained approximately 25% of their original phosphorylation at 0.2 – 0.25 mg chlorophyll/ml, corresponding to a liberation of 15 – 20% CF_1. After dilution up to 0.1 mg chlorophyll/ml the phosphorylation was completely uncoupled but led to a regeneration of 30 – 40% after readdition of the coupling factor. This tendency was found in a large

Table II. The influence of the chlorophyll concentration during the hypotonically extraction with PP on the reconstituting capacity of thylakoid membranes. The isolated thylakoids were suspended in 1 mM Hepes pH 7.4 and 0.5 mM PP as indicated (PP "+"). The chlorophyll concentration during hypotonic treatment is varied as indicated (mg Chl/ml). Cyclic phosphorylation was measured as described in Material and Methods. For reconstitution purified CF_1 was added in excess (CF_1 "+"). The release of CF_1 during hypotonic treatment was determined in the supernatant by the trypsin activated ATPase assay. The total amount of CF_1 corresponds to 458 µmol ATP hydrolyzed × mg chlorophyll^{-1} × hour^{-1}.

mg Chlorophyll/ml	PP	CF_1	Phosphorylation [µmol ATP/mg Chl×h]	Release of CF_1 [%]
	–	–	325	
0.11	+	–	0	32
	+	+	135	
	–	–	459	
0.23	+	–	129	16
	+	+	425	
	–	–	476	
0.44	+	–	452	5
	+	+	435	

number of experiments carried out over more than three years with plant material harvested from different seasons and grown in a climatic chamber or in the green house.

The lower degree of reconstitution with EDTA-extracted thylakoids and purified CF_1 was discussed to be due to the requirement of additional factors [26] and to the release of other membrane constituents [6]. For instance Shoshan and Shavit [6] found a significant reconstitution in low-chlorophyll incubated membranes only with their own extract whereas purified CF_1 alone was considerably less efficient or failed to reconstitute.

The ATP-formation after recombination of the extracted membranes with their corresponding supernatant and with purified CF_1 is shown in Table III and Fig. 5, respectively. Since the rate of cyclic phosphorylation was found to be higher after addition of CF_1, the requirement of other factors is excluded under the conditions used. The lower rate of phosphorylation after reconstitution with the supernatant is assumed to be due to a membrane damage as evidenced by the altered fatty acid pattern of the liberated lipids and to the release of free fatty acids [11]. Furthermore, higher amounts of the supernatant added to the membranes were often slightly inhibitory. As calculated from the amount of CF_1-protein which was required to saturate the

Table III. Reconstitution of photophosphorylation with the crude extract. Thylakoids were treated with PP as indicated in the legend of Table II. The reconstitution was carried out with the corresponding supernatant after centrifugation.

Thylakoids	Reconstitution excess of crude extract	Phosphorylation [μmol ATP/ mg Chl×h]
control	—	290
PP-extracted	—	0.2
PP-extracted	2 X	83
PP-extracted	5 X	85

reconstituted system with the purified coupling factor or the supernatant, the binding capacity of CF_1 was improved in the crude extract. Therefore, the lower degree of ATP-formation after reconstitution is suggested to be due to an impaired energy transfer rather than the rebinding of CF_1. This conclusion became even more evident by the different amount of added CF_1 which was required to saturate the phosphorylation and the 9-AAC fluorescence quench as shown in Fig. 5. The 9-AAC quench was recently discussed to be an indicator for the membrane energetization rather than for the proton gradient [16, 27]. The maximum value to saturate the 9-AAC quench was already reached at 0.2 mg CF_1 protein whereas the phosphorylation required approximately 1 – 3 mg CF_1 protein.

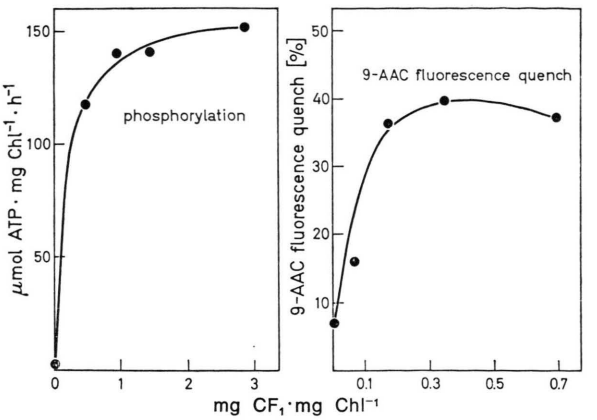

Fig. 5. Reconstitution of photophosphorylation and membrane energetization by purified CF_1. The reconstitution was carried out as described under material and methods.

These results led to the assumption that the lower degree of reconstitution is in the main due to an impaired energy transfer from the energized state of the membranes to the phosphorylating machinery. This conclusion is supported by the complete retention of CF_1 and of ATPase which are incapable to regenerate the phosphorylation [28].

The technical assistance of Miss M. Koch is gratefully acknowledged. This study was supported by a scholarship after the Graduiertenförderungsgesetz.

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