SYNTHESIS OF ADP BY ISOLATED "COUPLING FACTOR" FROM CHLOROPLASTS

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1. Introduction

The "coupling factor" responsible for the terminal steps of photophosphorylation in chloroplasts has been extracted from the chloroplast membranes [1, 2] and obtained in pure form by Vambutas and Racker [3]. When isolated from chloroplasts, the enzyme shows Ca^{2+}-dependent ATPase activity upon activation by heat or by trypsin treatment. The activation of Ca^{2+}-dependent or Mg^{2+}-dependent ATPase can also be obtained by treatment of the enzyme with dithiothreitol [4]. Recently Roy and Moudrianakis have shown that two molecules of ADP are bound per each 13 S enzyme complex [5], and that bound ATP is converted to bound ATP and AMP, however no free ATP and AMP are produced [5]. These authors have shown that the bound nucleotides can be released upon irreversible denaturation of the enzyme and presented evidence suggesting that the enzyme-bound AMP is the early phosphate acceptor of photosynthetic phosphorylation.

We have extracted the "coupling factor" from "class I" spinach chloroplasts by a slightly different method, and evidence is presented here that the isolated enzyme can catalyze the incorporation of inorganic phosphate into ADP. This activity is preserved through several purification steps including ammonium sulfate fractionation and sucrose gradient centrifugation. This finding indicates that the "energized state" of the enzyme is quite stable and can be utilized in the absence of membranes for phosphorylation.

2. Methods

"Class I" chloroplasts were prepared from freshly harvested spinach leaves as previously described [6]. The chloroplast suspension containing 5–10 mg chlorophyll/ml in 0.33 M sorbitol, 0.05 M HEPES* buffer pH 7.6, 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ was diluted 12-fold with ice cold 10 mM HEPES buffer, pH 8, 10 mM NaCl. The osmotically disrupted chloroplasts were sedimented at 0–3° by centrifugation at 30,000 *g* for 20 min and washed once. The crude chloroplast extract containing the "coupling factor" is then fractionated with enzyme-grade ammonium sulfate. The fraction sedimenting between 0.35 and 0.60 saturation, containing most of the activity, is dialyzed extensively against 10 mM HEPES pH 8. The small amount of denatured protein and ribosomes present are sedimented by centrifugation at 120,000 *g* for 60 min. At this stage of purification the enzyme contains adenylate kinase activity, NADPH diaphorase activity due to the chloroplast flavoprotein [7] and the "coupling factor". Fig. 1 shows an electron micrograph of the preparation obtained by negative staining with phosphotungstic acid. Further purification can be obtained by centrifugation of the enzyme in a sucrose density gradient (5–20% sucrose, w/v, in 10 mM HEPES pH 8.0), as shown in fig. 2. The ADP phosphorylating activity is found in the most dense fractions, still associated with some of the flavoprotein, while most of the latter is separated in the middle part of the gradient. Adenylate kinase activity is completely separated from the phosphorylation enzyme and is found in the upper

* HEPES: *N*-Hydroxyethylpiperazine-*N'*-2-ethanesulfonate.

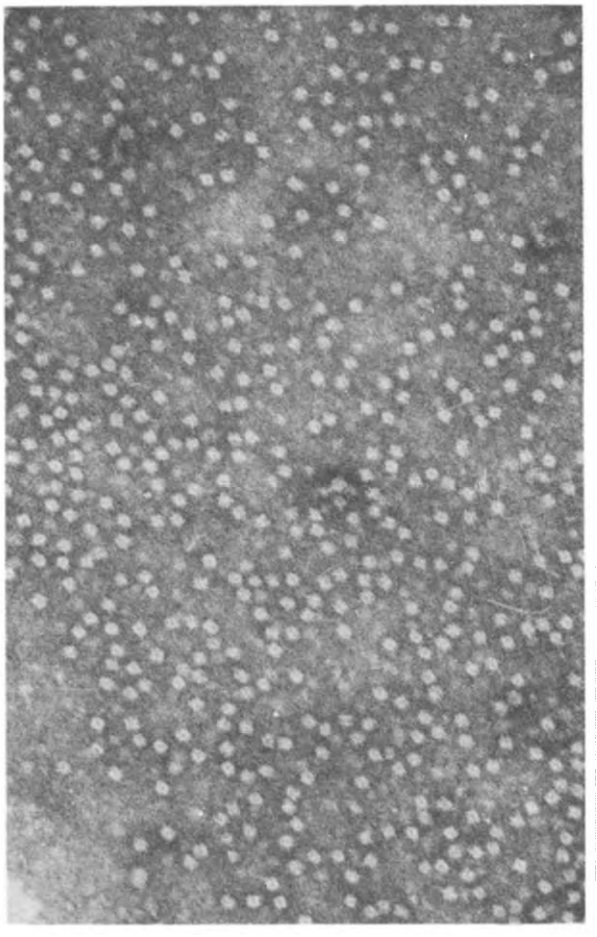

Fig. 1. Electron micrograph of the phosphorylating enzyme preparation negatively stained with phosphotungstic acid. Magnification: 200,000 ×.

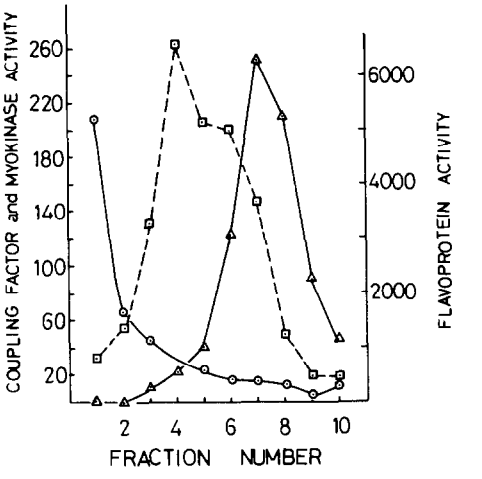

Fig. 2. Separation of coupling factor, flavoprotein and adenylate kinase on a sucrose density gradient. The preparation (0.5 ml, ammonium sulfate fraction, containing 21 units of A_{280}) was layered on a 5–20% sucrose gradient in 0.05 M HEPES buffer, pH 8.0, and centrifuged 24 hr in a Spinco SW50 rotor at 35,000 rpm, at 4°. The total volume of the gradient was 5.5 ml. (○—○—○) $^{32}P_i$ incorporation activity, in nanomoles/hr; (□—□—□) NADPH-diaphorase activity of the flavoprotein, in nanomoles/min; (△—△—△) adenylate kinase activity, in nanomoles NADPH/min (see Methods). Each fraction: 0.5 ml.

part of the gradient, as previously reported by Roy and Moudrianakis [5].

2.1. *Activity measurements*

The phosphorylation of ADP was measured by the incorporation of ^{32}P-labelled inorganic orthophosphate into organic compounds, according to the method of Avron [8]. The reaction mixture contained 60 mM HEPES buffer, pH 8.0, ADP, ^{32}P-labelled orthophosphate and $MgCl_2$ as indicated in the experimental section. The reaction was stopped by the addition of perchloric acid and the reaction products were measured enzymatically after removal of perchlorate as the potassium salt. G-6-P, ATP and ADP were determined (in a Cary 14 spectrophotometer, equipped with a 0.1 A slide-wire) as the NADPH produced by the sequential addition of G-6-P dehydrogenase, hexokinase and glucose, and adenylate kinase. The incorporation of $^{32}P_i$ into ATP and ADP was measured by absorption of these nucleotides on Norit A (activated). An aliquot was acidified and treated with Norit A. The radioactivity on the charcoal belongs to ATP + ADP. A second aliquot was treated with hexokinase and glucose previously to Norit A absorption, thus removing the γ-phosphate group of ATP and a third aliquot was similarly pre-treated with hexokinase, glucose and adenylate kinase to remove β- and γ-phosphates from ATP and β-phosphate from ADP. Appropriate zero time incubation samples were measured in all experiments. The diaphorase activity of the flavoprotein was measured as previously described [7]. Adenylate kinase was measured as the rate of NADPH formation in the presence of ADP, glucose, $MgCl_2$ and an excess of hexokinase and G-6-P dehydrogenase.

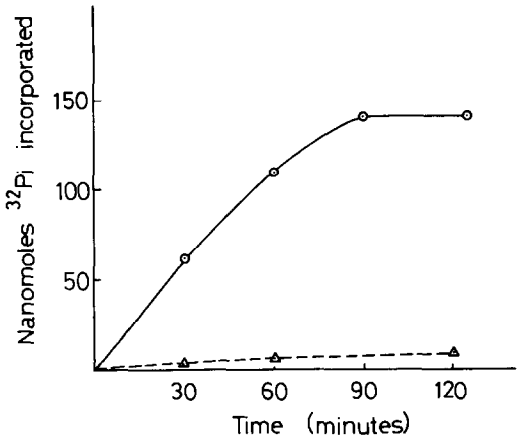

Fig. 3. Time-course of ADP phosphorylation by the coupling factor. Conditions: 0.06 M HEPES, pH 8.0; 1 mM ADP; 4 mM $MgCl_2$; 1.3 mM phosphate containing 200,000 cpm $^{32}P_i$; coupling factor (0.35–0.60 saturated $(NH_4)_2$ SO_4 fraction) equivalent to 8.4 units of A_{280}; temp: 32°. (⊙—⊙—⊙) Complete; (△- - △- - △) minus $MgCl_2$.

3. Results and discussion

The isolated "coupling factor" contains no membranes nor vesicles (see fig. 1) and incorporates $^{32}P_i$ into adeninucleotides (fig. 3) only in the presence of Mg^{2+}. The reaction is rather slow under these conditions, and comes to an end in 90–150 min.

The incorporation of $^{32}P_i$ is not due to the well-known ATP–$^{32}P_i$ exchange reaction because it is not, in contrast to the latter reaction, inhibited by ADP and, most important, it occurs in the absolute absence of ATP. This is shown in table 1, where the presence of hexokinase-glucose does not affect the $^{32}P_i$ incorporation, while it converts quantitatively ATP into G-6-P. Table 1 also shows that the amount of $^{32}P_i$ incorporated is identical to the amount of ATP produced (or G-6-P in the presence of hexokinase-glucose). However, ATP or G-6-P are produced in the same amounts and at the same rate whether or not P_i is present. Obviously under this condition no ^{32}P is incorporated into organic phosphate compounds (table 1). No $^{32}P_i$ is incorporated into the γ-phosphate group of ATP nor in G-6-P. The radioactivity incorporated is entirely present in the β-phosphate group of ADP, as it can be transferred, by treatment of the reaction product with adenylate kinase plus hexokinase, into gl_6–P. Added AMP does not serve as a phosphate acceptor.

Table 1
Stoichiometry of the phosphorylation reaction.

	(nanomoles formed / 150 min)							
Additions	P_i initial, nanomoles	^{32}P organic	G-6-P	G-6-^{32}P	ATP	ATP-$\gamma^{32}P$	ADP	ADP-$\beta^{32}P$
None	57	0.142	0.0	0.0	89	0.0	882	0.0
Glucose, 20 mM + Hexokinase	57	0.180	96.0	0.0	0.0	–	927	–
Inorganic phosphate 2450 nmoles	2500	94.0	0.0	0.0	97.0	0.0	868	104
Glucose, 20 mM + Hexokinase + P_i 2450 nmoles	2500	96.0	102.0	0.0	0.0	–	945	103

Conditions: HEPES buffer, 0.06 M, pH 8.0; 1 mM ADP; 4 mM $MgCl_2$; ^{32}P (carrier free) 350,000 cpm; coupling factor (adenylate kinase-free) containing 0.27 units of absorbance at 280 mm (ratio A_{280}/A_{260} = 1.71). The formation of organic $^{32}P_i$, ATP and G-6-P was linear during the reaction. ATP concentration was 0.00 at any time during incubation with hexokinase-glucose. Temperature: 32°. Volume 1 ml.

4. Conclusions

The findings reported here indicate that the phosphorylating enzyme isolated from chloroplasts ("coupling factor") is able to incorporate P_i into the β-phosphate group of ADP when supplied with ADP, phosphate and Mg^{2+}.

The net synthesis of the pyrophosphate bonds obviously requires the utilization of "high energy" chemical bonds (or an "energized state") which are necessarily present in the enzyme complex isolated from the membranes of chloroplasts and subjected to several steps of purification. The preparation is extensively purified, though some minor protein contaminants are still present. However, it contains no membranes nor vesicles of any kind (see fig. 1). (The amount of $^{32}P_i$ incorporated into ADP^{32} was found to vary, in 12 different preparations, from a maximum of 78 to a minimum of 17 nanomoles/mg of protein. Assuming a molecular weight of 340,000 for the 13 S enzyme [5], up to one mole of $^{32}P_i$ per 13,000 mol. wt. of protein would be incorporated).

The following reaction sequence is suggested as a working hypothesis:

$$(1)\ E^* + 2\ ADP \rightleftharpoons E^*_{ADP}{}^{ADP} \rightleftharpoons E^*_{ATP}{}^{AMP} \rightleftharpoons E^*AMP + ATP$$

$$(2)\ E^*AMP + {}^{32}P_i \rightarrow E{\cdot}AD^{32}P$$

(E* denotes the enzyme in the "high energy" state). The scheme accounts for the fundamental observations of table 1, namely: (a) $^{32}P_i$ incorporation into ADP^{32} is stoichiometrically equivalent to ATP formation (or G-6-P formation in the presence of hexokinase-glucose); (b) ATP (or G-6-P) formation is not dependent on phosphate but it occurs, independently of ^{32}P incorporation, at the same rate as this process. The binding of 2 ADP per enzyme molecule was previously reported by Roy and Moudrianakis [5]. These authors also suggested, on the basis of the distribution of radioactivity in the nucleotides bound to the "coupling factor" isolated from chloroplasts illuminated in the presence of pyocyanine and $^{32}P_i$, that enzyme-bound AMP is the primary phosphate acceptor [5]. If the $^{32}P_i$ incorporation reported here were due to ADP–$^{32}P_i$ exchange involving *free* ADP, one would expect the label to be found in the γ-phosphate of ATP subsequently formed, while this is not the case. Furthermore, an ADP–$^{32}P_i$ exchange reaction would be difficult to reconcile with the observed 1 to 1 stoichiometry of $^{32}P_i$ incorporation and ATP formation.

Indication for phosphorylating activity in isolated coupling factor from mitochondria has been recently reported by Fischer et al. [9]. Consistent with the above results, our data clearly show that indeed phosphorylating activity can be demonstrated in purified chloroplast coupling factor in the absence of vesicles or membrane fragments.

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