AMP IS CONVERTED TO ADP AND ATP IN THE MEDIUM BEFORE IT IS BOUND TO COUPLING FACTOR 1 IN ILLUMINATED SPINACH CHLOROPLAST THYLAKOIDS

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1. Introduction

Several lines of evidence suggest that AMP may play a role in photophosphorylation. Purified coupling factor 1 (CF_1) has a slow adenylate kinase-like activity [1,2]. Illumination of thylakoids in the presence of AMP and P_i causes ADP to be tightly bound to CF_1 [1,3]. It was suggested [1,3] that AMP phosphorylation to bound ADP may be the primary phosphorylation reaction and that the bound ADP transfers its β phosphate to medium ADP to form ATP and regenerate AMP. AMP also promotes a light-dependent $^{32}P_i$–ATP exchange [4] and together with arsenate relieves the inhibition of electron transport by ATP [5].

ADP and ATP in the medium exchange with adenylates bound to CF_1 in illuminated thylakoids [6–8]. The conversion of medium AMP to ADP bound to CF_1 is slow [3]. Some ADP and ATP could be formed from AMP during the illumination. The ADP and ATP in the medium could then exchange with the nucleotides bound to CF_1. The results presented here give support to this indirect pathway for the conversion of medium AMP to bound ADP.

2. Materials and methods

Spinach chloroplast thylakoids were isolated from market spinach [9] and were suspended in a medium which contained: 0.4 M mannitol, 0.02 M tris(hydroxymethyl)-methylglycine–NaOH (pH 8.25) and 0.01 M NaCl. Chlorophyll was determined spectrophotometrically [10]. All reactions were carried out at room temperature in a reaction mixture which contained, unless noted otherwise: 20 mM or 50 mM Tris-(hydroxymethyl)-methylglycine–NaOH (pH 8.25), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM potassium phosphate buffer, 0.05 mM pyocyanine, 0.02 mM [^{3}H]AMP (spec. radioact. ~40 cpm/pmol) and thylakoids equivalent to 0.2 mg chlorophyll/ml. Nucleotide binding to thylakoids was determined as in [8]. Nucleotides were separated by chromatography with either columns of polyethyleneimine cellulose [11] or on thin layers of this anion exchange material [12].

Hexokinase was desalted on a 1 × 15 cm column of Sephadex G-50 (medium) equilibrated with 25 mM sodium acetate buffer (pH 5.4) which also contained 50 mM glucose. Hexokinase activity was determined under the conditions used in the assay of nucleotide binding and conversion.

[^{3}H]AMP was purchased from New England Nuclear. About 95% of the radioactivity was present as AMP. In some experiments, [^{3}H]AMP, which had been purified to > 99% by chromatography on a polyethyleneimine-cellulose column [12], was used. This purification did not alter the results. Hexokinase, polyethyleneimine-cellulose and AMP were obtained from Sigma. Diadenosine pentaphosphate was obtained from Boehringer-Mannheim.

3. Results and discussion

3.1. *Conversion of AMP to ADP and ATP*

Small amounts of ADP are present even in well washed thylakoids. ADP is bound to CF_1 [6,13] and dissociates in part from the thylakoids upon illumination [6,7]. It could then be converted to ATP by

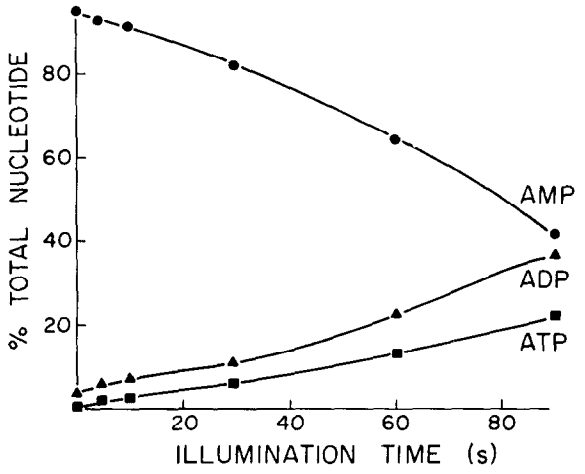

Fig.1. Conversion of AMP to ADP and ATP by illuminated thylakoids. Aliquots (25 μl) of the reaction mixture in section 2 were placed in polyethylene microcentrifuge tubes and illuminated for the times shown with about 2 × 10^6 ergs/cm^2/s of white light. The non-illuminated samples were incubated for 90 s. Trichloroacetic acid 10% (5 μl) was then added and the precipitated thylakoids were pelleted by centrifugation. To 20 μl supernatant fluids aliquots 10 μl solution containing 10 mM each of AMP, ADP and ATP was added. Nucleotides in 10 μl aliquots of these mixtures were separated by chromatography on thin layers of polyethyleneimine-cellulose [13].

photophosphorylation. Adenylate kinase may catalyze synthesis of more ADP from the added AMP and the newly formed ATP. These reactions could continue until much of the AMP is converted to ADP and ATP. As shown in fig.1, [^{3}H]AMP is slowly converted by illuminated thylakoids to [^{3}H]ADP and [^{3}H]ATP. The rate of the conversion increases with time as would be expected if the conversion follows the pathway outlined above. In 14 separate experiments performed under the conditions in the legend to fig.1, 28.7 ± 8.5% of the AMP was converted to ADP and ATP during 60 s illumination. A net synthesis of ADP and ATP from AMP and P_i, rather than just exchange, occurs. Thylakoids illuminated for 60 s in the presence of 50 μM AMP and 1 mM P_i catalyze the formation of 12.7 μM ADP and 4.3 μM ATP, concomitant with the disappearance of 17.1 μM AMP. Although the synthesis of ADP and ATP from AMP occurs in the absence of added P_i, it is sensitive to $HAsO_4^{2-}$. For example, without added P_i, light caused 20% of the [^{3}H]AMP to be converted into ADP and ATP in 60 s. In contrast, this value was only 3% in the presence of 2 mM $HAsO_4^{2-}$. The residual P_i content of the chloroplast preparations is ~0.05–0.07 μmol/mg chlorophyll. Since chlorophyll is 0.2 mg/ml, P_i would be present at 10–14 μM. These concentrations of P_i apparently suffice for photophosphorylation under conditions where the rate of phosphorylation is severely limited by the very low concentrations of ADP.

The light-dependent formation of ADP and ATP from AMP was completely inhibited when Mg^{2+} was omitted from the reaction mixture and 1 mM EDTA was added. Mg^{2+} (5 mM) restored the capacity of illuminated chloroplasts to convert AMP to ADP and ATP.

If ADP and ATP formation from AMP were the consequence of the combined action of photophosphorylation and adenylate kinase, it would be expected that this conversion would be inhibited by preventing the accumulation of ATP. To test this, the effect of hexokinase and glucose on the conversion was determined. As illustrated in fig.2, hexokinase (21 units/ml) totally blocked the conversion.

To further test the involvement of adenylate kinase in the conversion of AMP to ADP and ATP,

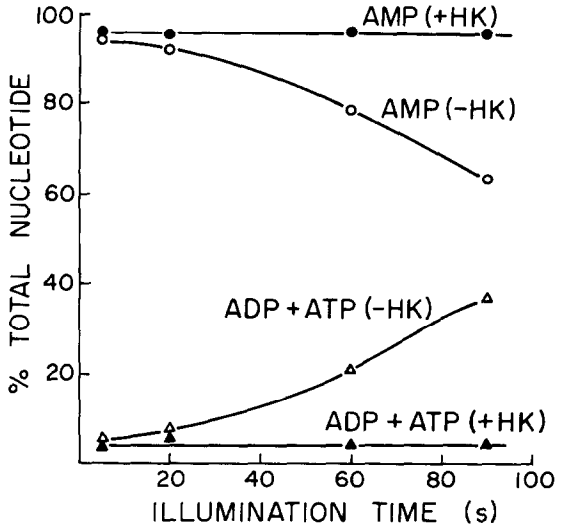

Fig.2. Effect of hexokinase (HK) and glucose on the conversion of AMP to ADP and ATP. This experiment was performed as in the legend to fig.1 except that 10 mM glucose was present in all samples and that hexokinase (21 units/ml) was added where indicated.

the effects of the adenylate kinase inhibitor, P^1,P^5-di(adenosine-5')pentaphosphate [14] were examined. The adenylate kinase activity of chloroplasts, assayed in the presence of 10 μM AMP and 33 μM ATP, was abolished by 10 μM diadenosine pentaphosphate. This concentration of the inhibitor also completely prevented the conversion of AMP to ADP and ATP, but had no effect on photophosphorylation.

Clearly then, AMP is converted to ADP and ATP. In view of the sensitivity of this conversion to $HAsO_4^{2-}$, to the absence of Mg^{2+}, to the action of hexokinase and to diadenosine pentaphosphate, it is probable that it occurs by a pathway which includes photophosphorylation of ADP followed by the phosphorylation of AMP by the newly formed ATP by adenylate kinase present in the chloroplast preparations. The rate of the conversion is slow (5–8 μmol/h/mg chlorophyll), probably because of the very low ADP concentrations and low rates of adenylate kinase activity. Adenylate kinase activity was assayed by following the conversion of [^{3}H]AMP to [^{3}H]ADP in thylakoids supplemented with ATP in the dark. With the low concentrations of AMP and ATP used (20 μM and 10–40 μM, respectively) it is difficult to assess the true initial rate of adenylate kinase activity since the reaction approaches equilibrium rather rapidly. However, even with only 10 μM added ATP and 20 μM AMP a rate of AMP conversion of about 5 μmol/h/mg chlorophyll was observed. In the presence of 40 μM ATP, this value was ~12. Exposure of thylakoids to hypotonic shock (dilution of the thylakoids to 0.1 mg chlorophyll/ml with 10 mM NaCl, followed by centrifugation after 10 min at 0°C) reduced the adenylate kinase activity by only 1/3rd. Thus, adenylate kinase in our thylakoid preparations is sufficiently active to participate in the conversion.

3.2. *Nucleotide binding to thylakoids*

Confirming results in [3], the binding of radioactive nucleotide to CF_1 in thylakoids is rather slow (fig.3). Also shown in fig.3 is the conversion of

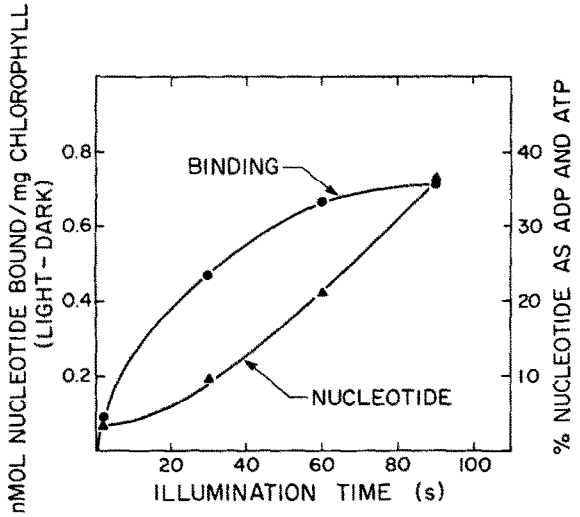

Fig.3. Time course of nucleotide binding. The reaction mixture (0.25 ml) contained about 5×10^4 cpm [^{14}C]sorbitol in addition to the other components. Binding was assayed by the silicone fluid microcentrifugation method [9]. The nucleotides present in aliquots of the suspending medium were determined as in the legend to fig.1.

Table 1
Effects of $HAsO_4^{2-}$, EDTA and hexokinase on nucleotide binding

Expt.	Additions or deletions	Nucleotide bound (60 s light – dark) (nmol/mg chlorophyll)	%AMP converted to ADP and ATP by 60 s illumination
1	$-P_i$	0.89	29.0
	$-P_i$ + 2 mM $HAsO_4^{2-}$	0.22	3.4
2	+ 5 mM Mg^{2+} + 1 mM EDTA	0.69	20.4
	– Mg^{2+} + 1 mM EDTA	<0.1	<1
3	+ 10 mM glucose	0.74	17.0
	+ 10 mM glucose + hexokinase	<0.1	<1
4	none (control)	0.79	23.7
	+ 10 μM diadenosine pentaphosphate	<0.1	<1

Nucleotide binding and AMP conversion were determined as in the legend to fig.3

AMP to ADP plus ATP. Assuming that no ADP or ATP was originally present in the thylakoid preparations, the concentrations of ADP and ATP together are 2 μM, 4.4 μM and 7.2 μM at 30 s, 60 s and 90 s illumination, respectively. At these concentration of ADP and ATP, there is considerable binding of these nucleotides to CF_1 in thylakoids [9]. $HAsO_4^{2-}$ has been shown [3] to prevent the binding of ADP to CF_1 in thylakoids illuminated in the presence of AMP and P_i. $HAsO_4^{2-}$, however, blocks the conversion of AMP to ADP and ATP in addition to the binding (table 1). Moreover, no significant binding of nucleotide or conversion of AMP takes place in thylakoids supplemented with EDTA to chelate endogenous cations.

More significant, however, is the finding that hexokinase and glucose as well as diadenosine pentaphosphate prevent both nucleotide binding and AMP conversion. If AMP and P_i were directly converted to bound ADP by CF_1, the binding would not be sensitive to the ATP trapping system or to an adenylate kinase inhibitor. Light-dependent ADP exchange was insensitive to diadenosine pentaphosphate even at 50 μM.

These results clearly show that the binding of ADP to CF_1 in thylakoids illuminated in the presence of AMP and P_i can not be taken as evidence that CF_1 catalyzes a direct phosphorylation of AMP. They do not, however, rule out this possibility. In some rapid mixing experiments, phosphate incorporation into ADP by illuminated thylakoids was more rapid than that into ATP [15], whereas in other experiments [16] little incorporation into ADP was detected. Whether AMP plays a role in photophosphorylation remains to be established.

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