

DOES AMP PARTICIPATE
IN PHOTOSYNTHETIC PHOSPHORYLATION?

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SUMMARY

Osmotically disrupted chloroplasts catalyze a rapid, light and AMP and ATP dependent $^{32}\text{P}_i$ incorporation into ATP. Light does not stimulate $[^{14}\text{C}]$ AMP incorporation into ATP in this system. AMP in the presence of P_i inhibits electron flow in a manner analogous to ADP inhibition in the absence of P_i . The inhibition of AMP + P_i is reversed on addition of ADP.

Previous evidence has indicated that AMP plays no essential role in photosynthetic phosphorylation. Chloroplasts do not phosphorylate AMP to ATP [1]. Yet, some ADP can be made from AMP. The total production is small, and the ADP remains attached to the membrane-bound energy-transducing coupling factor, CF_1 . Nevertheless, this small photophosphorylation of AMP accounts for conversion of 1 mole of AMP per mole of CF_1 into tightly-bound ADP [1]. This observation suggests that photophosphorylation of AMP to ADP may cease once the active sites on all CF_1 molecules are filled with tightly-bound ADP. A role for AMP in photosynthetic phosphorylation may also be inferred from the observation that in energized chloroplast membranes CF_1 bound ADP or ATP exchange their beta phosphates with exogenously added P_i [2]. Similarly Smith et al [3] have found rapid initial ADP labeling by $^{32}\text{P}_i$ after a 45 msec illumination time. We find that osmotically disrupted spinach or swiss chard chloroplasts in the presence of AMP, ATP and light catalyze a rapid $^{32}\text{P}_i$

Abbreviations: CF_1 , chloroplast coupling factor.
Tricine, N-tris (hydroxymethyl) methylglycine.
HEPES, N-2 Hydroxyethylpiperazine-N -2 ethanesulfonic acid.

incorporation into free ATP. Using different conditions, Avron [4] has observed a similar but very slow $^{32}\text{P}_i$ uptake by swiss chard chloroplasts. He attributed this P_i uptake to conversion of AMP + ATP to ADP by adenylate kinase followed by photophosphorylation of ADP to ATP. We find that adenylate kinase is relatively low in our chloroplast preparations and cannot account for our observed rapid $^{32}\text{P}_i$ uptake. Furthermore, light does not stimulate [^{14}C] AMP incorporation into ATP, and there is no net uptake of P_i .

Present reactions are abolished by antibody to CF_1 , and thus may reflect diverse catalytic roles of the three nucleotide binding sites [5] of CF_1 , as well as conformationally induced changes in enzymatic activities due to nucleotide binding at specific sites [5,6]. One possible way to interpret our data is that AMP + ATP dependent $^{32}\text{P}_i$ uptake might be due to rapid CF_1 -mediated photophosphorylation of AMP to AD^{32}P on a high-affinity site. The high rates, and the lack of light-dependent [^{14}C] AMP incorporation into ATP, might indicate that bound AD^{32}P rapidly transfers its beta ^{32}P to a second ADP bound at a different site on CF_1 to yield gamma labeled ATP. The ATP dependence of our $^{32}\text{P}_i$ uptake, and the lack of net incorporation of P_i , suggest that this second bound ADP may result from ATP-induced ATPase activity of CF_1 as proposed by Cantley & Hammes [5]. This explanation requires that AMP photophosphorylation result in ADP bound to a high-affinity site on CF_1 . We have tested this requirement by measuring effects of AMP + P_i on rate of electron flow. Two ADP sites of membrane-bound CF_1 have previously been shown to have opposite effects on electron flow. Binding to a high-affinity site (ADP alone) reduces electron flow, while binding to a low-affinity site (ADP + P_i) increases it [7,8]. We found AMP + P_i to inhibit electron flow, as would be expected if newly-formed ADP were tightly-bound to a high-affinity site. This AMP

Table I

Light-dependent $^{32}\text{P}_i$ Incorporation into Organic Phosphates
 micromoles $^{32}\text{P}_i$ esterified/hr/mg chlorophyll

	Experiment #1 (MV)	Experiment #2 (MV)	Experiment #2 (PMS)	Experiment #3 (PMS)
No additions	8	-	-	-
AMP	9	2.4	2.5	17
ADP	171	80	291	492
ATP	29	12	13	54
AMP + ADP	171	-	-	-
ATP + ADP	191	-	-	-
AMP + ATP	221	76	79	153
AMP + ATP (dark)	3	2	3	13

Each 2 ml reaction mixture contained 30mM Tricine, pH 8.1; 10mM NaCl; 4mM MgCl₂; 2mM KH₂³²PO₄ (4.5 X 10⁵ cpm); nucleotides at 1mM; and an electron acceptor; either 0.1mM methyl viologen (MV), or 0.025mM phenazine methosulfate (PMS). For Experiments #1 and 2, 50 micrograms of spinach chlorophyll, for Experiment #3, 32 micrograms of swiss chard chlorophyll. Incubation was 2 min at 22°C in a water bath. Saturating red light was obtained with an Agena photographic flood lamp filtered by 6cm water and Corning color glass filter #CS 2-62.

effect is dependent on P_i , reversed by addition of ADP (P_i being present), and inhibited by antibody to CF_1 .

MATERIALS AND METHODS

Osmotically shocked chloroplasts were prepared [8] and chlorophyll was measured [9] as previously described. The final resuspension medium contained 0.2 M sucrose, 5 mM HEPES, pH 7.65, 2 mM MgCl₂ and 0.05% defatted bovine serum albumin. Swiss chard chloroplast preparation and assay media were supplemented with 20 mM ascorbic acid. Preparation media also contained 0.1% defatted bovine serum albumin. $^{32}\text{P}_i$ incorporation into organic phosphates was determined as described by Sugino and Miyoshi [10], and modified by Jagendorf [11]. One ml of reaction mixture was denatured with 0.5 ml of 3% (w/v) ammonium molybdate in 0.67 N HCl and 1% redistilled triethylamine. After $\frac{1}{2}$ hr the precipitate was removed by centrifugation. 0.5 ml of the supernatant was mixed with 10 ml of aquasol and counted in a scintillation counter. Chromatography of nucleotides desalted by absorption on charcoal [2] (400 mg in 2 ml of water/1 ml reaction mixture) was done on precoated plastic sheets in 0.85 M LiCl and 1 mM EDTA pH 3.5. Nucleotide spots, localized by U.V. light, were scraped and nucleotides were eluted for 3-4 hrs in 1N HCl. Nucleotide concentration of eluates was determined at 259 nm and counted by liquid scintillation as for ^{32}P . Ferri-cyanide reduction and oxygen evolution were performed as previously described [12]. Sodium salts of AMP, ADP and ATP were purchased from Sigma (we detected no ADP contamination in this AMP); H₂³²PO₄, aquasol, from New England Nuclear; sodium salt of [¹⁴C] AMP from ICN; precoated plastic sheets, CEL 300 PEI, from Brinkmann Instruments; spectral grade LiCl, from Pierce Chemical.

Table IILight-dependent $^{32}\text{P}_i$ and $[^{14}\text{C}]$ AMP Incorporation into ADP and ATP

	Light cpm/spot	Sp. Act.	Dark cpm/spot	Sp. Act.
Experiment #1: AMP + ATP + $^{32}\text{P}_i$				
ADP	2,740	31,890	150	-
ATP	14,980	87,610	550	2,500
Experiment #2a: AMP + ATP + $^{32}\text{P}_i$				
ATP	2,258	30,268	68	839
Experiment #2b: $[^{14}\text{C}]$ AMP + ATP + P_i				
AMP	20,330	199,509	17,032	238,543
ATP	2,836	23,932	2,200	24,202

Reactions carried out as in Table I. Specific Activities (Sp. Act.) are given as cpm/micromole of nucleotide. Chloroplasts were inactivated after incubation by heating in a boiling water bath for 2 min. All subsequent steps were carried out in dim light until chloroplasts were removed by centrifugation. For chromatography, nucleotides were absorbed on charcoal, washed free of inorganic phosphate and then eluted with ethanol-ammonia (1:1). Experiment #1 performed with spinach chloroplasts and MV contained approximately 1.4×10^6 cpm $^{32}\text{P}_i$ /ml. Experiment #2a and b performed with swiss chard chloroplasts used in Table I, Experiment #3; Experiment #2a contained 1.3×10^6 cpm $^{32}\text{P}_i$ /ml. Experiment 2b, 4.5×10^5 cpm/ml. $[^{14}\text{C}]$ AMP.

In Experiment #1, dark counts were subtracted from light counts for specific activity calculations. AMP is not included in Experiment #1 because $^{32}\text{P}_i$ moved with the AMP spot. ADP spot was not detected in Experiment #2.

RESULTS

Table I gives the effect of nucleotides on $^{32}\text{P}_i$ uptake by spinach and swiss chard chloroplasts. AMP in combination with ATP induced $^{32}\text{P}_i$ uptake in spinach chloroplasts which ranged from 30% higher to 75% lower than $^{32}\text{P}_i$ uptake with ADP alone. Swiss chard chloroplasts (and fragments), catalyze AMP + ATP-dependent $^{32}\text{P}_i$ uptake¹, which is about 25% of $^{32}\text{P}_i$ uptake with ADP alone (Exp. 3). AMP concentrations from 0.1-1.0 mM did not alter the rate of $^{32}\text{P}_i$

¹Avron observed little or no $^{32}\text{P}_i$ uptake with swiss chard chloroplasts or their fragments using 4 mM AMP + 0.03 mM ATP in a 10 min. reaction period [4].

Table IIIEffect of AMP + P_i on Light-dependent O_2 EvolutionMicromoles O_2 /hr/mg chlorophyll

No additions	124
P_i	133
AMP	116
AMP + P_i	69
ADP	68

Each 5 ml reaction mixture contained 30mM Tricine, pH 8.3; 10mM NaCl; 4mM $MgCl_2$; 100 micrograms chlorophyll; 0.4mM $K_3Fe(CN)_6$; and where indicated: 1mM KH_2PO_4 ; 0.5mM AMP; 0.2mM ADP. All reactions were performed in saturating light.

uptake, but higher concentrations were inhibitory. Optimal concentration for ATP was about 0.5-1.0 mM. Antibody to CF_1 inhibited $^{32}P_i$ uptake induced by AMP + ATP.

The same swiss chard chloroplast preparation was used in Table I, Exp. 3 and Table II, Exps. 2a and b. Adenylate kinase activity of these chloroplasts was estimated as the AMP-induced increase in light dependent $^{32}P_i$ incorporation over a nonsaturating range of ADP (0.02-0.06 mM) in a 2 min. reaction². Adenylate kinase contributed 8 umoles of ADP/hr/mg chlorophyll, while the same preparation induced 153 umoles/hr/mg chlorophyll of $^{32}P_i$ uptake with AMP (1.0 mM) + ATP (1.0 mM) (Table I, Exp. 3). Enzymatic assay [13] of similarly prepared swiss chard chloroplasts for adenylate kinase gave 17 umoles of ATP/hr/mg chlorophyll. Our AMP + ADP assay method for adenylate kinase activity of spinach chloroplasts gave 10-15 umoles of ADP/hr/mg chlorophyll.

²We estimated the amount of additional ADP generated from AMP by adenylate kinase from the plot of $AT^{32}P$ produced vs ADP added in presence and absence of 0.5 mM AMP. AMP caused a constant, small increase in $^{32}P_i$ uptake.

Table IVEffect of ADP on Photosynthetic Control Exerted by AMP + P_i microequivalents $K_3Fe(CN)_6$ reduced/hr/mg
chlorophyll

No additions	207
ADP + P_i	294
ADP	165
P_i added 2 min later	303
AMP + P_i	154
ADP added 2 min later	291

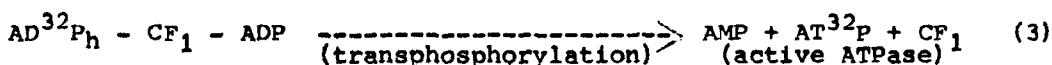
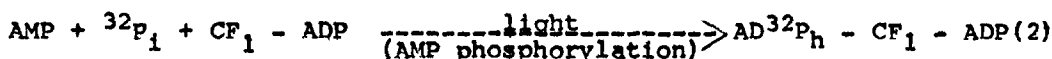
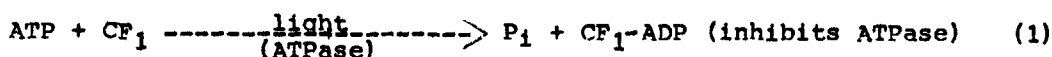
Reactions were carried out as in Table III, except: ADP concentration, 0.4mM; and 50 micrograms chlorophyll/5 ml.

Table II shows that the $^{32}P_i$ primarily labeled ATP, and to a smaller extent ADP. Light did not stimulate $[^{14}C]$ AMP incorporation into ATP (Exp. 2b) with swiss chard chloroplasts, though it occasionally caused slight stimulations with spinach chloroplasts. The dark incorporation of $[^{14}C]$ AMP into ATP by swiss chard chloroplasts amounted to about 77 umoles/hr/mg chlorophyll. Since light did not stimulate this reaction in the presence of P_i , it is likely that $[^{14}C]$ AMP was incorporated directly into ATP, without going through ADP. Such interconversions have been inferred from nucleotide levels in whole leaves [14].

Table III shows that electron flow was inhibited by AMP in the presence of P_i . Antibody to CF_1 abolished this effect of AMP + P_i . Swiss chard chloroplasts also show photosynthetic control (inhibition) of electron flow in the presence of AMP + P_i . Table IV shows that ADP completely reversed the AMP + P_i inhibition, restoring electron flow to that found with ADP + P_i .

DISCUSSION

The reaction sequence given below invokes known activities of membrane-bound CF_1 . This sequence is consistent with AMP and ATP-dependent rapid $^{32}P_i$ uptake into ATP in absence of ADP (Table I), with the catalytic role of AMP (Table II), with sensitivity of the reactions to antibody to CF_1 , and with no net uptake of P_i . ADP binding to a high-affinity site is indicated by the subscript h.



Substrate for transphosphorylation reaction (3) might also be produced by reversing the order of reactions (1) and (2).

We suspect that ATPase activity of CF_1 may be inhibited by some newlyformed ADP which remains bound to CF_1 , since $^{32}P_i$ uptake is slow in the presence of ATP alone (Table I). AMP might release any ADP-induced inhibition of ATPase, via reactions (2) and (3). The rate of $^{32}P_i$ uptake with AMP + ATP is sometimes limited when compared to $^{32}P_i$ uptake with ADP (Table I, Exp. #2 and #3). We have been unable to determine whether this limiting step might be our proposed ATPase reaction.

The AMP + P_i inhibition of electron flow is completely released by added ADP (Table IV). We suggest that an ADP-ADP interaction of both sites might form ATP and AMP, thereby releasing electron flow. Mukohata and Yagi [15] observed that the inhibition of electron flow induced by ATP was released by AMP + arsenate.

The present data suggest that CF_1 in its native environment may be capable of rapid AMP photophosphorylation, and also may be capable of rapid transphosphorylation of 2 ADP molecules to produce

ATP while recycling AMP. Under physiological conditions, free ADP might provide the second ADP which we may have experimentally provided through the ATPase activity of CF_1 . In this case it is conceivable that AMP is the primary phosphate acceptor in photophosphorylation.

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