# DOES AMP PARTICIPATE IN PHOTOSYNTHETIC PHOSPHORYLATION?

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## SUMMARY

Osmotically disrupted chloroplasts catalyze a rapid, light and AMP and ATP dependent $^{32}P_i$ incorporation into ATP. Light does not stimulate [$^{14}C$] 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 $^{32}P_i$ 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 $^{32}P_i$

Abreviations: $CF_1$, 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 $^{32}P_i$ 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 $^{32}P_i$ 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 $^{32}P_i$ 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 $^{32}P_i$ 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 $^{32}P_i$ Incorporation into Organic Phosphates

micromoles $^{32}P_i$ 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 MgCl; 2mM $KH_2{}^{32}PO4$ ($4.5 \times 10^5$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.

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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 $MgCl_2$ 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. $^{32}P_i$ 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 ½ 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 $^{32}P$. Ferricyanide 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); $H_3{}^{32}PO_4$, aquasol, from New England Nuclear; sodium salt of [$^{14}$C] AMP from ICN; precoated plastic sheets, CEL 300 PEI, from Brinkmann Instruments; spectral grade LiCl, from Pierce Chemical.

Table II

Light-dependent $^{32}P_i$ and [$^{14}C$] AMP Incorporation into ADP and ATP

| | Light | | Dark | |
|---|---|---|---|---|
| | cmp/spot | Sp. Act. | cpm/spot | Sp. Act. |
| | | Experiment #1: AMP + ATP + $^{32}P_i$ | | |
| ADP | 2,740 | 31,890 | 150 | - |
| ATP | 14,980 | 87,610 | 550 | 2,500 |
| | | Experiment #2a: AMP + ATP + $^{32}P_i$ | | |
| ATP | 2,258 | 30,268 | 68 | 839 |
| | | Experiment #2b: [$^{14}C$] 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 X $10^6$cpm $^{32}P_i$/ml. Experiment #2a and b performed with swiss chard chloroplasts used in Table I, Experiment #3; Experiment #2a contained 1.3 X $10^6$cpm $^{32}P_i$/ml. Experiment 2b, 4.5 X $10^5$cpm/ml. [$^{14}C$] AMP.

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

## RESULTS

Table I gives the effect of nucleotides on $^{32}P_i$ uptake by spinach and swiss chard chloroplasts. AMP in combination with ATP induced $^{32}P_i$ uptake in spinach chloroplasts which ranged from 30% higher to 75% lower than $^{32}P_i$ uptake with ADP alone. Swiss chard chloroplasts (and fragments), catalyze AMP + ATP-dependent $^{32}P_i$ uptake[1], which is about 25% of $^{32}P_i$ uptake with ADP alone (Exp. 3). AMP concentrations from 0.1-1.0 mM did not alter the rate of $^{32}P_i$

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

Table III

Effect of AMP + $P_i$ on Light-dependent $O_2$ Evolution

| | Micromoles $O_2$/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. reaction[2]. 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.

[2]We 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 IV

Effect 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.

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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.

$$ATP + CF_1 \xrightarrow[(ATPase)]{light} P_i + CF_1\text{-}ADP \text{ (inhibits ATPase)} \quad (1)$$

$$AMP + {}^{32}P_i + CF_1 - ADP \xrightarrow[(AMP\ phosphorylation)]{light} AD{}^{32}P_h - CF_1 - ADP \quad (2)$$

$$AD{}^{32}P_h - CF_1 - ADP \xrightarrow[(transphosphorylation)]{} AMP + AT{}^{32}P + CF_1 \quad (3)$$
$$(active\ ATPase)$$

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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