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INTERACTION OF A METHYLENE DIPHOSPHONATE ANALOG OF ADP WITH PHOTOSYNTHETIC MEMBRANES OF CHLOROPLASTS.

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SUMMARY

The ADP analog α,β-methylene adenosine 5'-diphosphate was photophosphorylated to α,β-methylene adenosine 5'-triphosphate by spinach chloroplasts. The phosphorylation of the analog was not due to nucleotide diphosphokinase activity. The ADP analog was binding to the nucleotide binding sites on the photosynthetic membranes as well as to the solubilized coupling factor protein. Binding of ADP to the photosynthetic membranes was decreased in the presence of the ADP analog indicating a direct interaction of the ADP analog with the ADP binding sites on the thylakoids.

INTRODUCTION

The details of the mechanism of ATP formation during photosynthetic phosphorylation are not yet known. Roy and Moudrianakis (1, 2) proposed a mechanism in which photosynthetic formation of ATP is brought about by transphosphorylation of two coupling factor-bound ADP molecules to form AMP and ATP. The AMP would then be rephosphorylated in an electron transport dependent step to form coupling factor bound ADP. This would act as the high energy intermediate to phosphorylate another ADP molecule to ATP.

We used the ADP analog α,β-methylene adenosine 5'-diphosphate to determine whether ADP undergoes the transphosphorylation to AMP and ATP during photophosphorylation. In this analog pyrophosphate oxygen of ADP is replaced by a methylene bridge ($-CH_2-$). This makes the analog resistant to hydrolysis or transfer of the terminal phosphoryl group. The analog is therefore unable to undergo the transphosphorylation reaction.

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Abbreviations: AOPCP, α,β-methylene adenosine 5'-diphosphate; AOPCPOP, α,β-methylene adenosine 5'-triphosphate; TES, tris (hydroxymethyl) methylaminoethane sulfonic acid; CF_1, coupling factor 1.

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We found that AOPCP was photophosphorylated to the corresponding α,β-methylene analog of ATP in a light-dependent reaction catalyzed by spinach chloroplasts (3). Photophosphorylation of AOPCP resembled phosphorylation of ADP in requiring $MgCl_2$ and phenazine methosulfate to obtain full activity (4). The two reactions had a similar pH optimum and a similar sensitivity to Dio-9 and to NH_4Cl(4). These similarities suggested, that photophosphorylation of AOPCP occurred by the same type of reaction as phosphorylation of ADP.

However, the photophosphorylation of AOPCP was about ten times slower than phosphorylation of ADP. Also, the ATP analog α,β-methylene adenosine 5'-triphosphate was a poor substrate for ATP-Pi exchange and its hydrolysis was not stimulated by light and dithiothreitol nor was it inhibited by Dio-9(5). These observations raised the question whether AOPCP was phosphorylated directly by the thylakoid-bound coupling factor, or whether its phosphorylation was indirect, mediated by other enzymes such as nucleotide diphosphokinases. We tried to resolve this question in the present study by following the possible contribution of nucleotide diphosphokinase activity to phosphorylation of AOPCP and by measuring the binding of AOPCP to the ADP-binding sites present on the thylakoid-bound coupling factor.

MATERIALS AND METHODS

Photophosphorylation and nucleotide diphosphokinase activity in chloroplasts.

Spinach chloroplasts were prepared and their photophosporylation capacity was measured as described previously (3, 4). Nucleotide diphosphokinase activity was assayed essentially as described by Avron (6): chloroplasts were first incubated in light for 10 min in presence of 2 mM ADP, 3 mM sodium phosphate containing ^{32}P and all the other components used in the photophosphorylation assay (4). After switching off the lights, 2 mM AOPCP was added and the incubation continued in the dark for 10 min. After denaturation with 0.2 ml 60% trichloroacetic acid, the nucleotides were separated on polyethyleneimine-cellulose sheets, using 2M LiCl for development (7). The distribution of radioactivity between [^{32}P] ATP and [^{32}P] AOPCPOP spots on the chromatograms was measured and from it the nucleotide diphosphokinase activity was calculated.

Nucleotide binding to thylakoids.

Light-induced binding of [U-^{14}C] ADP and of [^{3}H] AOPCP was measured as described by Strotmann et al. (8). Chloroplasts were prepared by homogenizing

spinach leaves in a Vir-tis 45 homogenizer with a medium containing 300 mM sucrose and 10 mM sodium pyrophosphate pH 7.4 (9). The homogenate was filtered through cheesecloth and centrifuged for 5 min at 3000 g. The chloroplast pellet was washed once with the homogenizing medium and three times with a solution containing 40 mM sucrose and 25 mM TES-NaOH pH 8.0.

Light-induced nucleotide binding was measured in a medium containing: 25 mM TES-NaOH (pH 8.0), 40 mM sucrose, 5 mM $MgCl_2$, 90 µM phenazine methosulfate, [U-^{14}C] ADP or [^{3}H] AOPCP in concentrations indicated in Fig. 1 and Fig. 2 and chloroplasts containing 0.5 to 0.6 mg chl. Total incubation volume was 1.0 ml, incubation time 5 min at 20° C and 100,000 lux of white light. After incubation, chloroplasts were centrifuged down at 8,000 g for 5 min and the pellet was washed four times with 1 ml of 40 mM sucrose containing 25 mM TES-NaOH (pH 8). The resulting thylakoid pellet was suspended in 0.5 ml of the washing medium and radioactivity of a 50 µl aliquot of this suspension was counted in an Isocap 300 liquid scintillation counter.

Nucleotide binding to the solubilized CF_1.

CF_1 was extracted from chloroplasts that were preilluminated for 5 min at 20° C and 100,000 lux of white light in a medium containing 40 mM sucrose, 25 mM TES-NaOH (pH 8.0) and 90 µM phenazine methosulfate. The CF_1 was extracted from these chloroplasts by the method of Strotmann et al. (9). The solubilized CF_1 was concentrated to 500 µg/ml by pouring the CF_1 solution into a dialysis tubing placed in polyethylene glycol 20,000. The binding of [^{3}H] AOPCP to the soluble CF_1 was carried out by incubating the concentrated CF_1 extract containing 150 µg protein with a medium of 40 mM sucrose, 5 mM $MgCl_2$, 25 mM TES-NaOH (pH 8) and 1 to 10 µM [^{3}H] AOPCP. Total incubation volume was 0.5 ml and the incubation time was 2 hrs at 37° C (10). The amount of protein-bound nucleotide in this incubation mixture was measured by the technique of Penefsky (11): a 1 ml syringe was filled with Sephadex G-50 fine preswollen in 40 mM sucrose containing 25 mM TES-NaOH (pH 8). 0.1 ml of the above incubation mixture was applied onto this Sephadex column and centrifuged at 200 g for 2 min. The radioactivity was measured in 50 µl of the eluate obtained by the centrifugation.

Chlorophyll and protein determination.

Chlorophyll content of chloroplasts was determined by the method of Arnon (12) and protein was measured according to Sedmak et al. (13).

Chemicals.

AOPCP was purchased from Miles Laboratories and tritium labelled by Amersham. [U-^{14}C] ADP was purchased from Amersham, ADP and TES from Sigma. All other chemicals were from Fisher Scientific Co. Sephadex G-50 fine was purchased from Pharmacia and polyethyleneimineimine-cellulose sheets from Brinkmann.

RESULTS AND DISCUSSION

Chloroplast nucleotide diphosphokinase could catalyze a transphosphorylation: ATP + AOPCP $\longrightarrow$ ADP + AOPCPOP. To check whether this reaction is responsible for the observed formation of AOPCPOP, rather than direct photophosphorylation of AOPCP to AOPCPOP, we measured the rates of photophosphorylation and of nucleotide diphosphokinase activity in chloroplast preparations that were

Table 1: Photophosphorylation and nucleotide diphosphokinase activity in washed chloroplasts.

Chloroplast preparation (times washed with 10 mM NaCl)	Photophosphorylation Pi esterified (μmol/mg chl/hr)		$\frac{AOPCP}{ADP}$ ratio	Nucleotide diphosphokinase AOPCPOP formed (μmol/mg chl/hr)
	ADP	AOPCP		
0	749	66	0.088	49
2	904	70	0.077	6
6	819	62	0.076	6

washed 0, 2 or 6 times with 10 mM NaCl, to remove soluble proteins (Table 1). Photophosphorylation was measured with ADP or AOPCP as substrate. It is evident from Table 1, that washing of chloroplasts with 10 mM NaCl did not remove any protein, or other factor, required for phosphorylation of AOPCP, since the ratio of the phosphorylation rates for AOPCP:ADP remained constant. On the other hand, when the nucleotide diphosphokinase ability to form AOPCPOP was measured in these chloroplast preparations, the unwashed chloroplasts contained nucleotide diphosphokinase activity but this activity was decreased to a very low level in the 2 times and 6 times washed chloroplasts that were still capable of photophosphorylating AOPCP (Table 1). This indicated, that nucleotide diphosphokinase activity was not responsible for the observed photophosphorylation of AOPCP.

Since the phosphorylation of AOPCP was not caused by the nucleotide diphosphokinase, the ADP analog AOPCP should interact directly with the photophosphorylation system present in the chloroplast membranes. AOPCP should therefore bind to the thylakoids and it should compete with ADP for the ADP-binding sites that are present on the CF_1 protein of the thylakoid membranes. AOPCP binding to thylakoids was studied using [^{3}H]-labelled AOPCP. It can be seen in Fig. 1, that there was binding of [^{3}H] AOPCP to thylakoids when these were incubated with [^{3}H] AOPCP and all the other components required for light-induced electron transport. Having established, that

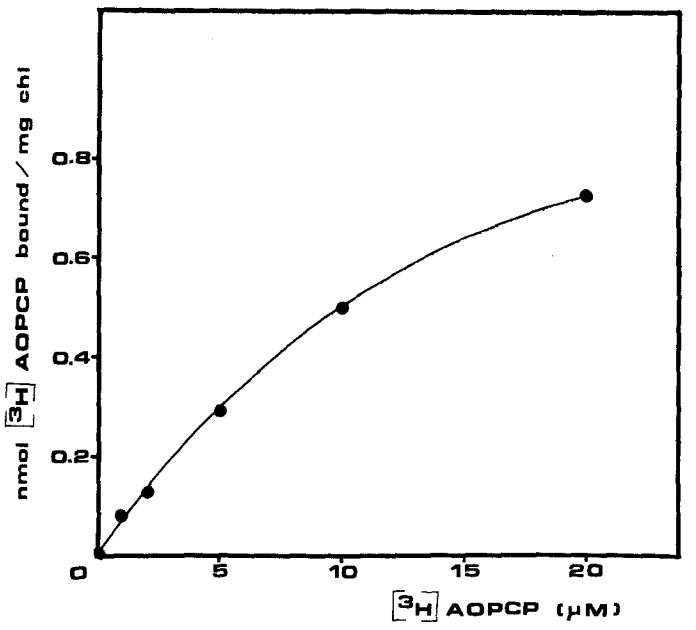

Fig. 1: Binding of [^{3}H] AOPCP to thylakoids as a function of [^{3}H] AOPCP concentration.

AOPCP did bind to thylakoids, we wanted to find out, whether AOPCP did bind at the sites, where normally binding of ADP takes place. This we studied by following light-induced binding of [^{14}C] ADP to thylakoids in presence or absence of AOPCP. The binding curves for [^{14}C] ADP binding to thylakoids show, that the binding of [^{14}C] ADP to the photosynthetic membranes decreased in the presence of AOPCP (Fig. 2), indicating, that ADP and AOPCP both bound to the same sites on the thylakoids.

Since AOPCP was binding to thylakoids (Fig. 1), and since the binding sites responsible for nucleotide binding to photosynthetic membranes are known to be localized on the coupling factor protein (14), we tested, whether AOPCP binds to the CF_1 protein, when this is solubilized from the thylakoids. The results in Table 2 show, that the solubilized spinach CF_1 did bind [^{3}H] AOPCP, indicating, that AOPCP did interact directly with the enzyme responsible for catalyzing photophosphorylation in the chloroplasts.

Our results show, that the ADP analog AOPCP interacts directly with the

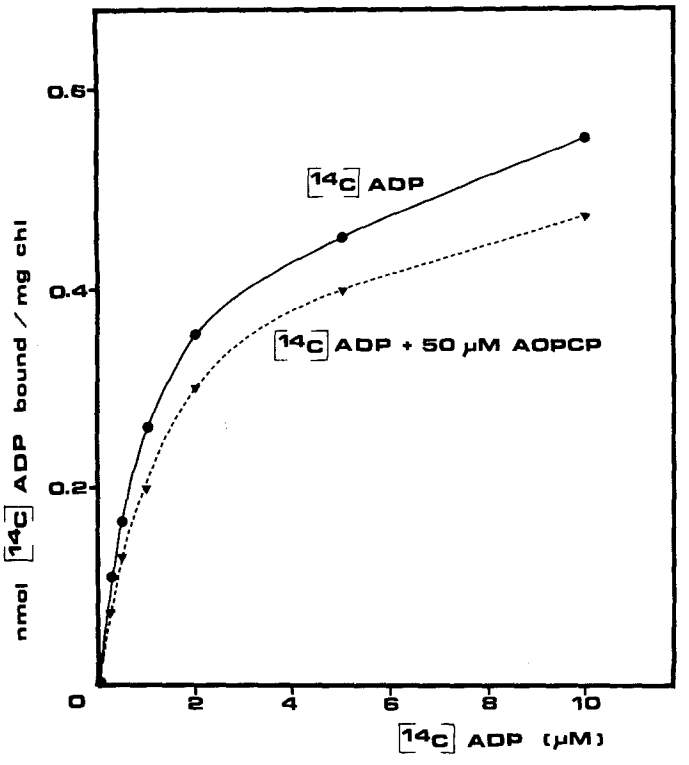

Fig. 2: Binding of [^{14}C] ADP to thylakoids as a function of [^{14}C] ADP concentration. Solid line shows binding of [^{14}C] ADP when no AOPCP was present while the dotted line shows binding of [^{14}C] ADP when 50 μM AOPCP was included in the incubation medium.

photophosphorylation system of spinach thylakoids, just like ADP, and that its phosphorylation is not due to the presence of nucleotide diphosphokinase activity in the chloroplasts. We can conclude, therefore, that substrate ADP

Table 2: Binding of [^{3}H] AOPCP to the solubilized CF_1

[^{3}H] AOPCP (μM)	[^{3}H] AOPCP bound (nmol/mg protein)
1	0.054
5	0.090
10	0.226

does not undergo a transphosphorylation to AMP and ATP. The results indicate that substrate ADP is directly photophosphorylated to ATP.

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