Medium ADP and Not ADP Already Tightly Bound to Phylakoid Membranes Forms the Initial ATP in Chloroplast Phosphorylation

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SUMMARY: Measurements are reported on the source of the ADP and γ-phosphoryl moieties of the initial ATP formed when chloroplast thylakoid membranes are energized by an acid-base transition. Millisecond mixing and quenching experiments demonstrate that most or all of the initial ATP, formed in amounts considerably less than the amount of CF_1-ATPase present, arises from medium ADP and medium P_i. With no or low amounts of added medium ADP, the tightly-bound ADP present in thylakoid membranes is released to the medium on energization, then subsequently forms ATP. These results rule out the possible conversion of the ADP tightly-bound to CF_1-ATPase to tightly-bound ATP as a step in the main pathway of chloroplast phosphorylation. Less complete experiments indicate a similar behavior of tightly-bound ADP of submitochondrial particles.

INTRODUCTION

ADP and ATP tightly-bound to isolatable ATPases of energy-transducing membranes from a variety of sources (1,2,3) have been proposed to be intermediates in net ATP synthesis. Yamamoto et al. (2) suggested that phosphorylation of a tightly-bound ADP is the first step in ATP synthesis by R. rubrum chromatorphores. Roy and Moudranakis (4) proposed that AMP and P_i first form a tightly-bound ADP which then forms ATP in a subsequent transphosphorylation reaction. Slater et al. (1) and Harris and Slater (5) suggested the possibility that tightly-bound ADP and medium P_i may form tightly-bound ATP, with energization necessary for release of the ATP (1). Lutz et al. (3) reported incorporation of $^{32}P_i$ into a tightly-bound ATP as a possible initial step in chloroplast phosphorylation. Energization of chloroplasts by exposure to light or an acid-base transition resulted in the labeling of the tightly-bound nucleotides with labeled nucleotides and P_i of the medium (5,6). None of the above experiments, however, established that reactions or release of the tightly-bound nucleotides occurred with sufficient rapidity to qualify as intermediates in the main path of ATP synthesis or were involved in the formation of the initial ATP formed.

Recent millisecond mixing and quenching experiments of Yamamoto and Tonomura (7) and Smith et al. (8) established that ADP is the initial detectable acceptor

of P_i in ATP synthesis by chloroplast synthesis, but did not suffice to identify whether the tightly-bound ADP or added medium ADP was the initial phosphoryl acceptor. Experiments reported in this paper demonstrate that medium ADP is the initial acceptor.

METHODS

Materials, millisecond mixing and quenching and analyses were essentially as described elsewhere (8). The amount and radioactivity of ADP was determined in the first 3 ml of the 60 mM HCl eluent, but washing with about 20 ml more was necessary to remove all detectable [^{3}H]ADP from the ATP fraction subsequently eluted with 1 M HCl.

Chloroplast thylakoid membranes with tightly-bound [^{3}H]ADP were obtained essentially as described by Harris and Slater (5). Typical preparations contained 0.7 to 10. mole of [^{3}H]ADP per mole of CF_1-ATPase, taking the amount of ATPase as 1.3 nmoles/mg chlorophyll (9).

Beef-heart submitochondrial particles were prepared as described by Beyer (10). To remove any free nucleotides they were passed through a Dowex AG-1-X_4 column equilibrated with a solution containing 250 mM sucrose, 10 mM Tris-Cl at pH 7.5.

RESULTS AND DISCUSSION

Origin of the initial ATP formed during the acid-base transition of chloroplast thylakoids. The washed thylakoids contain tightly-bound ADP and ATP but little or no P_i. Measurement of the ratio of $^3H/^{32}P$ in the initial ATP formed as compared to that of medium [^{3}H]ADP and $^{32}P_i$ (taken as 1.0) indicates that the initial ATP arises as follows:

A ratio < 1; tightly-bound ADP is phosphorylated by medium P_i.

A ratio = 1; medium ADP is phosphorylated by medium P_i.

A $^3H/^{32}P$ ratio > 1; medium ADP is phosphorylated by tightly-bound ADP or ATP.

ATP formation was limited to only a few molecules per molecule of CF_1-ATPase present, by either addition of medium ADP about equimolar with the amount of CF_1 present (hand-mixing 5-50 seconds incubations) or with considerably higher concentrations of [^{3}H]ADP but with millisecond mixing and quenching. The latter method gives a more critical test.

Experiments with limited ADP. Results given in Table 1 show that the $^3H/^{32}P$ ratio observed in the initial ATP formed was considerably less than 1. The ratio approaches 1 if sufficient medium [^{3}H]ADP is added to allow formation of ATP in considerable molar excess over CF_1. Adenylate kinase, which could give rise to a $^3H/^{32}P$ ratio $>$ thus, does not interfere. Similar results were also

TABLE 1

Labeling of ATP from Medium [^{32}P]P_i and Increasing Amounts of Medium [^{3}H]ADP Using Chloroplasts Energized by Acid-Base Transition

Chloroplast thylakoids (900 µg chlorophyll/ml) were suspended in 0.5 ml of 5 mM succinate and 5 mM $MgCl_2$ at pH 3.8 at 30°C. After 30 sec they were mixed with an equal volume of a solution of pH 8.2 containing 100 mM Tris-Cl, 2 mM [^{32}P]P_i (spec. act. 93250 cpm/nmole), 5 mM $MgCl_2$ and amounts of [^{3}H]ADP (spec. act. 120,000 cpm/nmole) as indicated. The reactions were quenched after 4 sec with 1 ml of 2 M perchloric acid. The labeled nucleotides were separated as described in the Methods.

Moles [^{3}H]ADP added per Mole CF_1	Moles [^{3}H]ATP formed per Mole CF_1	Moles [^{32}P]ATP formed per Mole CF_1	Relative Ratio $^{3}H/^{32}P$ in ATP*
0.0	-	0.6	-
0.5	0.2	0.8	0.25 or 0.3
1.0	0.5	1.1	0.45 or 0.5
2.0	1.0	1.7	0.59 or 0.6
3.1	1.6	2.1	0.76 or 0.8
4.1	2.1	2.5	0.84 or 0.8
8.1	3.5	3.6	0.97 or 1.0
25.4	10.7	10.0	1.07 or 1.1
40.7	13.7	12.2	1.12 or 1.1

*Ratio $^{3}H/^{32}P$ in ADP and P_i of reaction medium taken as 1.0.

obtained with submitochondrial particles (11), and led to the suggestion that the phosphorylation of a tightly-bound ADP might be the first step in the formation of medium ATP (11). However, if the dissociation of bound ADP that occurs on chloroplast energization (5,6) is relatively rapid the released ADP would lower the specific activity of the medium [^{3}H]ADP and a $^{3}H/^{32}P$ ratio of < 1 could be found. Experiments showed that the acid-base transition but not just exposure to acid would indeed cause release of most of the tightly-bound [^{3}H]ADP particularly in the presence of added medium ADP. Another approach was thus necessary.

Experiments with higher ADP concentration and with millisecond mixing and quenching. Results of an experiment with approximately 100 moles of added [^{3}H]

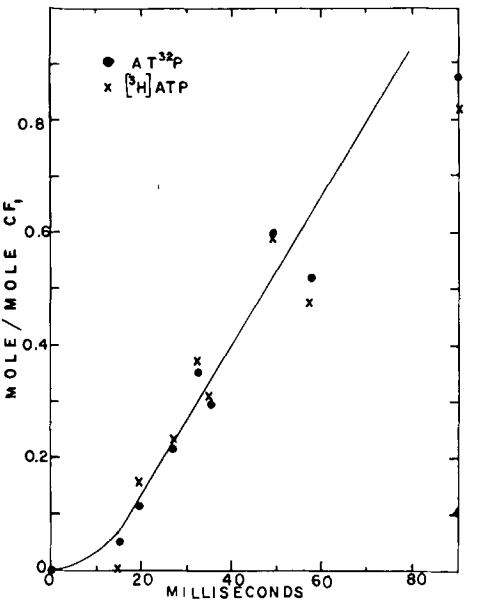

Figure 1

Labeling of ATP from Medium $[^{32}P]P_i$ and Medium $[^{3}H]$ADP in Acid-Base Transition of Chloroplast Thylakoids Using Rapid Mixing and Quenching

Chloroplast thylakoids (250 µg chlorophyll/ml) exposed in 0.5 ml at pH 3.8 to 5 mM succinate, 5 mM NaCl and 2.5 mM EDTA for 30 seconds were rapidly mixed with an equal volume of a solution at pH 8.2 containing 5 mM $MgCl_2$ 2 mM $[^{32}P]P_i$ (spec. act. 8.2 x 10^4 cpm/nmole), 100 mM Tris and 7.0 µM $[^{3}H]$ADP (spec. act. 1.7 x 10^5 cpm/nmole). The quenching was at the indicated time with an approximately equal volume of 1 M perchloric acid containing 5 mM EDTA. The labeled nucleotides were measured as described in the Methods section.

ADP per mole of CF_1-ATPase are given in Fig. 1. The results show that within reasonable experimental error the ratio of ^{3}H to ^{32}P in the initial ATP formed (considerably < 1 per CF_1) is the same as that in medium P_i and medium ADP.

As a confirmatory test experiments were performed with chloroplast thylakoids containing tightly-bound $[^{3}H]$ADP, but with unlabeled ADP in the medium. Results shown in Fig. 2 demonstrate that the initial ATP formed arose from medium $^{32}P_i$ and unlabeled medium ADP.

Results such as given in Figures 1 and 2 demonstrate that all or nearly all of the initial ATP formed by chloroplast thylakoid membranes in the acid-base transition arises from medium ADP and medium P_i.

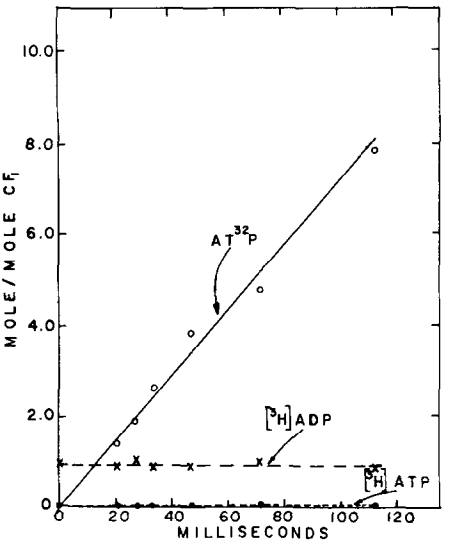

Figure 2

Lack of Preferential Phosphorylation of Tightly-Bound [^{3}H]ADP During the Acid-Base Transition

Chloroplast thylakoids containing 0.73 mole tightly bound [^{3}H]ADP *per mole of* CF_1 were prepared as described in the Methods section. The labeled thylakoids (408 µg chlorophyll/ml) were incubated in 0.5 ml of 5 mM succinate, 5 mM $MgCl_2$ at pH 3.8 for 30 sec., then rapidly mixed at 25° C with an equal volume of a solution containing 100 mM Tris-Cl, 5 mM $MgCl_2$, 2 mM [^{32}P]P_i (spec. act. 110,000 cpm/nmole) and 1 mM ADP at pH 8.2. The reaction was quenched at the indicated times by rapid mixing with 1 ml 2 M perchloric acid. Adenine nucleotides were isolated from the perchlorate extracts and measured as described in the Methods section. Observed values for [^{3}H]ATP formed reported in the figure were corrected for the small amount of [^{3}H]ATP found in a zero-time blank.

The incorporation of $^{32}P_i$ into tightly-bound adenine nucleotides without added medium ADP. $^{32}P_i$ is incorporated into tightly-bound ADP and ATP when chloroplast thylakoids energized by light (5) or the acid-base transition (8) in the absence of added adenine nucleotides, but incorporation is much slower than the initial rate of ATP synthesis (8). Such incorporation might result from a dissociation of ADP to the medium prior to ATP formation.

To test for this, experiments were conducted with and without added pyruvate kinase and phosphoenolpyruvate to trap any ADP released. Results in Table 2 demonstrate that the pyruvate kinase trap markedly decreases appearance of tightly-bound [^{3}H]ADP into ATP. Thus the [^{3}H]ADP was released to the medium.

TABLE II

Bound ADP Release and Phosphorylation
by Energization of Chloroplast Thylakoids
in Presence of P_i and Absence of Added ADP

Chloroplast thylakoids with 1.05 mole [^{3}H]ADP tightly-bound per mole CF_1 were prepared as described in the Methods section. In expt. 1 these thylakoids (200 µg chlorophyll) were incubated at 30°C and pH 3.8 in 0.5 ml of a solution containing 5 mM succinate and 5 mM $MgCl_2$. After 30 sec they were mixed with an equal volume of 2 mM [^{32}P]P_i (spec. act. 198,000 cpm/nmole), 100 mM Tris-Cl, 5 mM $MgCl_2$ at pH 8.2. Phosphoenolpyruvate and pyruvate kinase or glucose and hexokinase were present as indicated. The reactions were quenched by addition of 1 ml 2 M perchloric acid 10 sec later. In expt. 2 thylakoids (200 µg chlorophyll) were incubated in light (125 mW/cm^2) for 2 min at 30°C in 1 ml of a medium at pH 8.0 containing 25 mM Tris-Cl, 2.5 mM NaCl, 1.25 mM $MgCl_2$, 15 µM phenazine methosulfate, 1 mM [^{32}P]P_i (spec. act. 220,000 cpm/nmole). Glucose and hexokinase or phosphoenolpyruvate and pyruvate kinase were present as indicated. The reactions were quenched by addition of 1 ml 2 M perchloric acid. Adenine nucleotides were measured as described in the Methods section.

Conditions	[^{32}P]ATP/CF_1	[^{3}H]ATP/CF_1	[^{32}P]Glucose 6-phosphate/CF_1
	mole/mole	mole/mole	mole/mole
Experiment 1			
No pH jump	0	0.03	-
pH jump + 1.5 mg pyruvate kinase	0.16	0.15	-
pH jump + 1.5 mg pyruvate kinase + 5 mM phosphoenolpyruvate	0.05	0.32	-
pH jump + 1.5 mg hexokinase + 50 mM glucose	0.06	0.06	0.22
Experiment 2			
Light	0.23	0.20	-
Light + 1.5 mg pyruvate kinase + 5 mM phosphoenolpyruvate	0.015	0.25	-
Light + 1.5 mg hexokinase + 50 mM glucose	0.08	0.08	1.33

With a hexokinase-glucose trap present, the [^{3}H]ATP formed disappears as glucose 6-phosphate is formed (Table II). Clearly the ATP formed in absence of added ADP is accessible within 2 minutes to hexokinase and thus does not remain tightly-bound to the thylakoids.

TABLE III

Bound ADP release by energization of submitochondrial particles in absence of added ADP.

Beef heart submitochondrial particles with free nucleotides removed were added to a reaction mixture to give final concentrations, at pH 7.5 and 30° of 100 mM Tris, 50 mM sucrose, 3 mM $MgCl_2$, 20 mM succinate, 2.5 mM $^{32}P_i$, 5 mg of protein/ml, and with or without added 4 μM ADP or 1.5 mg pyruvate kinase per ml and 5 mM phosphoenolpyruvate.

Time	ADP added	Pyruvate kinase and phosphoenol-pyruvate added	[^{32}P]ATP formed/F_1	[^{32}P]ADP formed F_1
min			mole/mole	mole/mole
1	+	-	0.79	0.10
2	+	-	1.91	0.50
3	+	-	3.07	1.01
1	-	-	0.11	0.01
2	-	-	0.26	0.07
3	-	-	0.42	0.19
1	-	+	0.023	0.001
2	-	+	0.057	0.004
3	-	+	0.102	0.010

When medium ADP is present, as in the experiments reported in Fig. 2, the release of the tightly-bound ADP upon energization might be rapid. Unpublished findings show that this is indeed the case, and indicate that the tightly-bound ADP might occupy a catalytic site.

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