ON THE ROLE OF MEMBRANE-BOUND ADP AND ATP IN PHOTOPHOSPHORYLATION IN CHLOROPLAST MEMBRANES

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1. Introduction

The role of CF_1 firmly-bound nucleotides in photophosphorylation is of current and widespread interest and importance; especially as regards the proposal that conformational changes of the membrane-bound protein result in altered binding affinities for the substrates, ADP, P_i and ATP [1,2]. In addition, a light-dependent and uncoupler-sensitive exchange of nucleotides into membrane-bound CF_1 was recently discovered by Harris and Slater [3]. The relationship between this type of exchange reaction and ATP formation in chloroplasts was further investigated by Strotmann et al. [4] and Magnusson and McCarty [5]. Energization of thylakoid membranes in the presence of labeled ADP or ATP resulted in the incorporation of ADP into CF_1. The nucleotide bound to CF_1 in this manner appears for the most part to be associated with one of the two larger subunits of CF_1.

Recently, we reported on the light-dependent phosphorylation of CF_1-bound ADP and attempted to correlate this activity with the phosphorylation of free ADP [6]. Using short term illumination ($\geq$ 3 s), about one mole of bound ADP per mole of CF_1 was phosphorylated to yield an equivalent amount of [γ-^{32}P]ATP.

These studies have been further extended using short-term ($\geq$ 30 ms), saturating illumination to attempt to compare the kinetics characteristic of phosphorylation of bound and free ADP. In this communication, it is shown that the rate of phosphorylation of bound nucleotide:

(1) Is much lower than that observed with free ADP.

(2) Is dependent upon the electron carrier used, as observed also with normal photophosphorylation.

These results suggest that the bound nucleotide does not exist as an active enzyme–substrate complex with CF_1 (in vivo) which functions as the initial acceptor of P_i in the process of ATP formation.

2. Materials and methods

Chloroplasts were isolated from fresh market spinach leaves by conventional procedures, washed three times by resuspension in 0.4 M sucrose–1 mM tricine, pH 8.0, and centrifugation and finally resuspended in this same medium. Chlorophyll content was estimated according to Arnon [7].

Reaction mixtures for phosphorylation contained in a final volume of 1 ml the following components (μmol): tricine, 20; KCl, 20; $MgCl_2$, 5; $^{32}P_i$, 0.5 (containing about 4×10^7 cpm); phenazine methosulfate or pyocanine, 0.03–0.06, and thrice-washed chloroplasts containing about 100 μg chlorophyll. Samples were placed in small Pyrex test tubes (approx. 7.5 mm i.d.) and illuminated individually with a beam of strong white light from a 500 W projector lamp (Sylvania DAK). The intensity of the focused white light filtered through an infrared-absorbing lens was about 4.5×10^6 ergs cm^{-2} sec^{-1}. The duration of illumination was controlled by an electromagnetic shutter (model 225–OA3 Vincent Associates, Rochester, NY) operated in conjunction with an electronic timer set to provide an effective shutter opening time of 30 ms or longer. The actual exposure time was routinely checked with

a photodiode connected to a storage oscilloscope. Upon extinction of illumination, the reaction was terminated by trichloroacetic acid (TCA) addition to a final concentration of 6%. The estimated time for addition and mixing was about 200 ms. No significant ATPase activity was detected by introducing dark intervals (1 s and longer) before TCA addition. Radioactivity incorporated into the organic phosphate fraction was determined as described [6]. Each determination is the average of 2–4 samples. Hexokinase (50 units) and glucose (10 mM) were used where indicated. ^{32}P-labeled glucose-6-phosphate was estimated after chemical hydrolysis with 1 N HCl at 100°C for 10 min. Defatted bovine serum albumin (BSA) and hexokinase were purchased from Sigma Chemical Co.

3. Results

The product of photophosphorylation of membrane-bound ADP was earlier shown to be [γ-^{32}P]ATP [6]. The purpose of the present experiments was to evaluate kinetically the possibility that this reaction serves as an intermediate step in the overall mechanism of ATP formation. The rates of phosphorylation of membrane-bound ADP, using different catalysts and shortterm, saturating illumination is shown in fig.1. The reaction is essentially complete after 1 s. Both the total extent and the initial rate depend upon the electron transport carrier used. However, the initial rates observed are significantly lower than the rate of phosphorylation observed with saturating amounts of free ADP. The apparent K_m for ADP, determined under these conditions, is about 8 μM. No cooperativity effect was observed in the presence of low concentrations of free ADP.

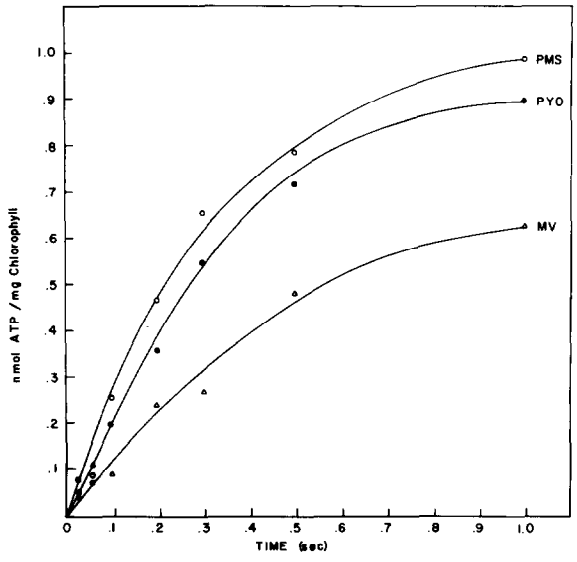

Fig.1. Rate of phosphorylation of membrane-bound ADP. Reaction mixtures were as described in Materials and methods. Phenazine methosulfate, pyocyanine or methyl viologen, 60 μM were used.

As shown in table 1, a 40-fold increase in rate was obtained by providing saturating concentrations of ADP. Thus, it is safe to conclude that the membrane-bound ADP behaves kinetically similar to, and is indistinguishable from, free ADP. Regarding the possible presence of membrane-bound P_i, this could be estimated by providing a pre-illumination period prior to $^{32}P_i$ addition and measurement of light-induced ATP formation. In separate experiments it was determined that no significant hydrolysis of ATP occurs during the intervening dark interval. Thus, any decrease in the amount of ADP phosphorylated (during the second illumination) could be attributed to the utilization of bound P_i during the pre-illumination period. Using this experimental approach, the concentration of membrane-bound P_i was estimated to be no greater than 10^{-8} M; that is, a concentration indistinguishable from the normal P_i contamination in our solutions.

During the course of these studies, we observed that

Table 1
Phosphorylation of membrane-bound and free ADP

ADP/CF_1 Ratio[a]	Rate[b]
1.1[c]	91
2.2	132
5.0	236
21.0	4128

[a] Molar ratio of CF_1 to chlorophyll is 1:860
[b] Nanomoles of ATP formed per milligram of chlorophyll per minute
[c] Endogenous ADP only

Reaction mixtures and assays as described in Materials and methods except that ADP was added as indicated and the illumination time was 0.5 s.

hypotonic treatment of chloroplasts reduced the amount of ADP, membrane-bound, that could be phosphorylated. We subjected therefore the thrice-washed membrane preparation to additional hypotonic washes and measured their capacity to phosphorylate bound ADP. In separate phosphorylation experiments with free ADP, it was shown that the washing procedure did not remove significant amounts of CF_1. Whereas the capacity to phosphorylate bound ADP was strongly affected by the hypotonic treatment, the capacity for phosphorylation with free ADP was not seriously affected (table 2). One can apparently remove more than 75% of the bound ADP without significant effect on the rate of free ADP phosphorylation. These results support the conclusion that the membrane-bound ADP probably does not participate as such in the formation of ATP.

The following experiment confirms our previous results [6] that, in energized membranes, a significant amount of the ATP formed appears to be membrane-bound (fig.2). The presence of increasing amounts of hexokinase (and glucose) in solution, together with energized chloroplasts synthesizing ATP, did not convert all the ATP to glucose-6-phosphate. The amount of ATP unavailable to the hexokinase 'trap' represents about 50% of that formed in the absence of hexokinase. Thus, in energized membranes, part of the newly formed ATP is probably bound to CF_1.

Table 2
Phosphorylation of bound and free ADP in hypotonically-treated chloroplasts

Pre-treatment	Additions	ATP Formation[a]	Rate[b]
Isotonic	None	1.1 (100)	–
	+ADP	137.2	494 (100)
Hypotonic	None	0.11 (10)	–
	+ADP	112.0	403 (81)

[a]Nanomoles per milligram of chlorophyll
[b]Micromoles of ATP formed per milligram of chlorophyll per hour

Thrice-washed chloroplasts were isolated as described [6]. Control chloroplasts were obtained by subjecting the thrice-washed chloroplast preparations to an additional wash in the sucrose–tricine medium, centrifugation at 8000 × *g* for 20 min and resuspension in this medium. Hypotonically-treated chloroplasts were obtained by subjecting the thrice-washed preparation to two additional washes in a medium containing: tricine, 10 mM, pH 8.0; KCl, 10 mM; $MgCl_2$, 215 mM, and BSA, 2 mg/ml. During hypotonic treatment, the chlorophyll concentration was 30–40 μg/ml. Pellets were collected by centrifugation at 8000 × *g* for 20 min and resuspended finally in the sucrose–tricine medium. Phosphorylation was assayed as described in Materials and methods by illumination for 1 s in the presence of 60 μM pyocyanine. Where indicated, 250 μM ADP was added. Numbers in parentheses represent percent of corresponding control.

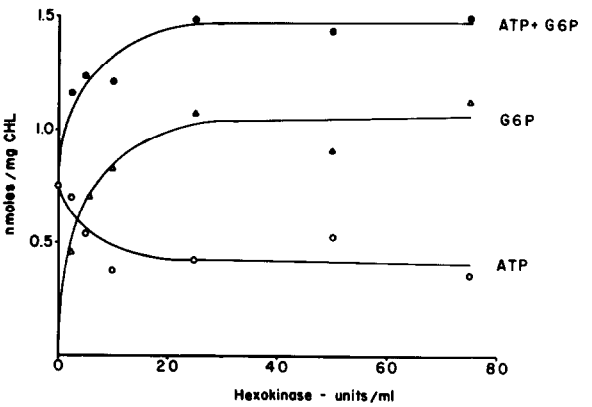

Fig.2. Formation of membrane-bound ATP. Reaction mixtures were as described in Materials and methods. ATP + G6P: total $^{32}P_i$ incorporated. G6P: non-hydrolyzable phosphates. ATP: hydrolyzable phosphates.

4. Discussion

The use of short-term, saturating illumination to induce phosphorylation of membrane-bound and free ADP allows for kinetic characterization of these two phosphorylation reactions. The lower initial rates of membrane-bound ADP phosphorylation may simply reflect the lower steady-state concentration of the active enzyme–substrate complex between CF_1 and ADP. The increase in phosphorylation rate observed by increasing ADP concentration in solution follows the usual Michaelis-Menten behavior. That is, the absence of a positive cooperativity effect appears to rule out a change in binding or release of membrane-bound nucleotide with consequent modification of the rate of phosphorylation.

The amount of membrane-bound ADP, determined directly or indirectly [3,6] is about equimolar with

CF_1. However, chloroplast membranes depleted of most of their membrane-bound ADP can still phosphorylate free ADP to ATP with initial rates comparable to control chloroplasts. Thus, it would appear that the ADP bound to CF_1 in de-energized membranes may first be released from the membrane-bound CF_1 upon energization. Indeed, membrane-bound ADP was shown to exchange rapidly with medium ADP in an energy-dependent reaction [8]. Using a rapid mixing technique and energization by an acid–base transition, it was proposed that the initial acceptor of P_i is medium ADP, a mechanism compatible with the data presented herein.

Although we have not performed direct determinations of membrane-bound P_i, our results indicate that significant amounts of membrane-bound P_i cannot participate in the phosphorylation of ADP. On the other hand, the formation of membrane-bound ATP in energized membranes seems firmly established. Thus, the formation of ATP on the membrane-bound enzyme from ADP and P_i in solution requires energy and depends upon electron transport in a manner analagous to that of normal photophosphorylation. These experiments do not rule out the possibility that upon energization there is a change in the binding affinities of P_i and ADP. They do indicate, however, that in the energized state the rate-limiting step may be the release of ATP. Upon de-energization, ATP is released and rebinding of ADP to the same or another site occurs. This is supported by the recently reported isolation of a labeled CF_1–ADP complex from thylakoid membranes energized in the presence of labeled ATP [5] and by the exchange reactions between medium and membrane-bound nucleotides [3,4]. The roles of this ATP bound to energized membranes, as well as of the nucleotides bound to de-energized membrane-bound CF_1 are not yet clarified and new experimental approaches may be required to provide satisfactory answers.

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