ROLE OF TIGHT NUCLEOTIDE BINDING IN THE REGULATION OF THE CHLOROPLAST ATP SYNTHETASE ACTIVITIES

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Received 12 July 1980

1. Introduction

Isolated thylakoid membranes have a high rate of ATP synthesis but only very low rates of ATP hydrolysis and P_i–ATP exchange [1]. Enhanced rates of ATP hydrolysis and P_i–ATP exchange can be obtained without affecting the rate of ATP synthesis by energization of the membranes in the presence of thiol reagents [2,3]. Illumination of the membranes for several minutes elicits maximal ATPase and P_i–ATP exchange activities [2,3] while lower than maximal activities are obtained by energization with short light flashes [4], short continuous illumination [5] or an acid–base transition [6]. Addition of ADP to activated chloroplasts membranes before ATP, the substrate for the hydrolytic or P_i–ATP exchange activities, was shown to enhance the decay of the activated state and to inhibit these activities [7]. The effect of ADP on the decay of the activated state of the ATPase was explained on the basis of the permeability of ADP and P_i across the membrane and their relative concentration gradients.

The membrane-bound ATPase complex contains, in addition to the catalytic site(s) for ATP synthesis and hydrolysis, tight nucleotide-binding sites where nucleotide-binding is very strong and not rapidly reversible [8–11]. The exchange of nucleotides from the tight nucleotide-binding sites depends upon energization but occurs rather slowly which suggests that these nucleotide-binding sites are not involved in the catalytic process of ATP synthesis or hydrolysis [12–14]. A correlation between the energy-dependent release of tightly-bound ADP and the activation of the ATPase was reported in [5].

Here we report on the binding of ADP to the tight binding site(s) on the ATPase, the inactivation of the ATPase and the inhibition of the P_i–ATP exchange and ATP hydrolysis. The conversion of the latent enzyme to the active conformation appears to involve conformational changes of the protein, in addition to the release of bound ADP, while reactivation of the ADP-inhibited form seems to depend on the rapid release of tightly-bound ADP. We propose that the interconversion between non-functional and functional conformations of the membrane-bound ATPase is controlled by the release and binding of nucleotides to the tight binding sites on the enzyme.

2. Materials and methods

Chloroplasts were isolated from fresh market lettuce leaves by conventional procedures. Thrice washed chloroplasts were prepared as in [15]. Chlorophyll content was estimated according to [16]. [2-^{3}H]ADP was purchased from Amersham, and [γ-^{32}P]ATP was synthesized from $^{32}P_i$ and ADP, and purified on Dowex 1-X8.

P_i–ATP exchange, ATPase and [^{3}H]ADP binding activities were all assayed under the same conditions. Reaction mixtures contained in 1.0 ml final vol. the following components (μmol): Tricine (pH 8.0), 20; NaCl, 20; $MgCl_2$, 10; dithiothreitol, 10; phenazine methosulfate, 0.03; P_i, 5; ATP, 5; and chloroplasts containing 50–70 μg chlorophyll (chl). An incubation mixture (0.9 ml) containing the above given components except for P_i and ATP, was stirred continuously and illuminated for 3 min with a beam of strong

Abbreviations: Tricine, tris (hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DTT, dithiothreitol

Elsevier/North-Holland Biomedical Press

white light (80 000 lux) filtered through a water layer. Immediately after illumination or after the indicated time in the dark, P_i and ATP were added (0.1 ml). The reactions were allowed to proceed in the dark for 5 min and terminated by the addition of trichloroacetic acid to 3% final conc. The reaction mixtures contained the following labeled compounds: for P_i–ATP exchange, $^{32}P_i$ (3×10^6 cpm) for ATPase [γ-^{32}P]ATP (5×10^5 cpm) and for ADP binding, [^{3}H]ADP (1×10^6 cpm). Binding of ADP was initiated by addition of 5 μM [^{3}H]ADP to activated chloroplasts. After incubation in the dark for the time intervals indicated, reactions were terminated by addition of ATP and P_i as in the other assays, followed by the addition of 5 μM FCCP, 30 s later. Bound [^{3}H]ADP was determined as in [14]. $^{32}P_i$ incorporated or liberated were determined by the isobutanol benzene extraction method [17].

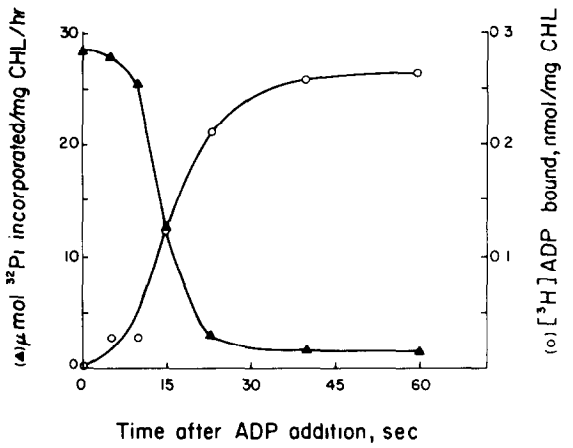


Fig.1. Binding of ADP and inhibition of the P_i–ATP exchange reaction. Thrice washed chloroplasts containing 55 μg chl. were activated and assayed as in section 2. ADP or [^{3}H]ADP (5 μM) were added immediately after the light was turned off. ATP and P_i were added at the time indicated.

3. Results

Addition of ADP to chloroplasts which were preactivated by illumination in the presence of dithiothreitol, results in the inhibition of the P_i–ATP exchange and Mg^{2+}-ATPase activities (table 1). Inhibition of 50% was obtained by ADP at <1 μM. ATP hydrolysis was assayed under the same conditions as the P_i–ATP exchange, namely, in the presence of P_i. Since the release of $^{32}P_i$ from [γ-^{32}P]ATP reflects both exchange and hydrolysis, the net rate of hydrolysis was obtained by substracting the rate of ^{32}Pi incorporation into ATP from the total rate of $^{32}P_i$ released. The inhibition by ADP was prevented if enough ATP was added together with ADP. Addition of ATP in the dark, after interaction with ADP

Table 1
Reversibility of the inhibition by ADP

Chloroplast treatment	P_i–ATP exchange	ATP hydrolysis
	μmol . mg chl^{-1} . h^{-1}	
1 Activated	21.2	29.5
2 (1) + ADP	0.9	6.6
3 (2) + illuminated	23.3	–
4 (3) + dark	1.2	–

Chloroplasts were activated and assayed as in section 2. ADP (5 μM) was added immediately after illumination and P_i + ATP after 1 min. Where indicated a second illumination (3 min) or a dark incubation period (1 min) were interposed

occurred, did not reverse the inhibition even at concentration ratios of 1000:1. However, re-energization of the ADP-inhibited chloroplasts by a second illumination, followed by the immediate addition of ATP, reversed the inhibition by ADP. Omitting the addition of ATP reinhibited the reaction. These observations suggest that binding of ADP to tight nucleotide binding sites may be the direct cause for the inactivation process. As shown in fig.1, binding of [^{3}H]ADP to the activated membranes is well correlated with the inactivation of the enzyme and the inhibition of P_i–ATP exchange activity. Since ADP is added to activated membranes in the post-illumination step, the binding of ADP probably corresponds to the slow dark phase of nucleotide binding, as described [13] for untreated chloroplast membranes.

Full reactivation of ADP-inhibited chloroplasts was reached by re-illuminating the membranes. The release of bound [^{3}H]ADP and the re-appearance of P_i–ATP exchange activity occur rapidly ($t_{1/2} < 1$ s) (fig.2). The amount of ADP bound to the ATPase is higher than that shown by the binding of [^{3}H]ADP (fig.1,2) due to the incomplete release of tightly bound nucleotides by energization [13]. The amount of [^{3}H]ADP bound after 5 s illumination (fig.2) reflects the amount of ADP that remains bound to the ATPase in the light. As expected, the binding of ADP by the activated membrane-bound ATPase did not affect the capacity to catalyze phosphorylation

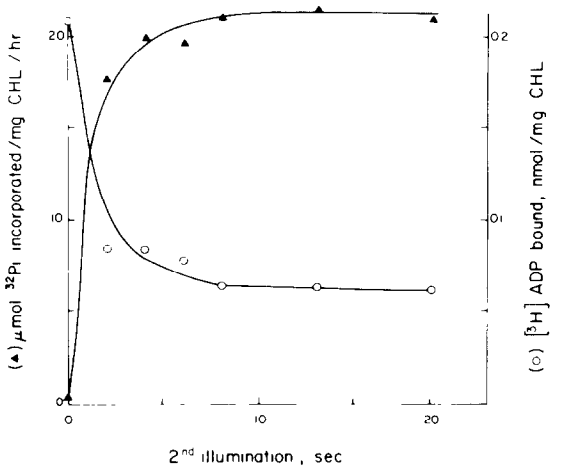


Fig.2. Release of [^{3}H]ADP and reappearance of the P_i–ATP exchange activity. Reaction conditions and assays as in fig.1 and section 2. After 1 min in the dark, the [^{3}H]ADP-labeled membranes were re-illuminated as indicated (2nd illumination). P_i and ATP were added immediately upon turning the light off.

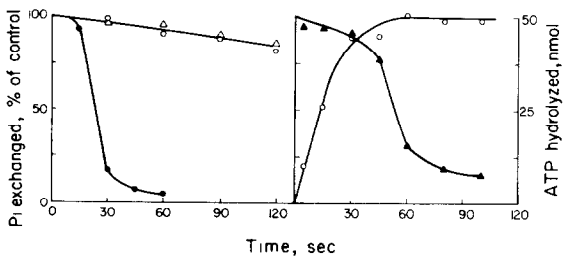


Fig.3. Inactivation of the P_i–ATP exchange by ADP formed by hydrolytic cleavage of ATP. Reaction mixtures and assays were as in section 2. Unlabeled or [γ-^{32}P]ATP, at the concentrations indicated, were added immediately after energization. After incubation in the dark for the indicated time, $^{32}P_i$ and ATP were added for measurement of the P_i–ATP exchange. For ATPase activity reactions were terminated by addition of 0.1 ml 30% trichloroacetic acid. (a) P_i–ATP exchange activity; (•) 10 μM ATP; (△) 250 μM ATP; (○) no additions. (b) P_i–ATP exchange activity (△) and ATP hydrolysis (○). The activated membranes were preincubated with 50 μM ATP. Control activities were 25–40 μmol P_i exchanged . mg chl^{-1} . h^{-1}.

(not shown). Phosphorylation requires continuous illumination that induces the rapid release of tightly bound ADP [14]. As shown in fig.3a, interaction of the activated membranes with ATP also appears to inhibit the P_i–ATP exchange activity. This inhibition depends on the concentration of ATP added and develops as the incubation time is prolonged. Fig.3b shows that the inactivation is not due to ATP but to ADP, formed by hydrolysis of ATP by the activated enzyme. The inactivation appears to depend both on the concentration of ATP remaining and on the rate of ADP binding to the enzyme (see fig.1). Maximal inactivation was obtained after complete hydrolysis of the ATP added and after a time interval of ~30 s that parallels the time required for maximal binding of ADP.

4. Discussion

The inhibition of ATP hydrolysis and P_i–ATP exchange activities by ADP seems to depend on the binding of ADP to the nucleotide tight binding site(s) in the activated state of the membrane-bound ATPase (fig.1). It is unlikely that the inhibition results from the binding of ADP to the catalytic site on the enzyme, since K_i-values for the competitive inhibition of the P_i–ATP exchange and ATP hydrolysis by ADP are much higher, 720 and 38 μM, respectively [3,18]. The inhibition by ADP could be prevented by the simultaneous addition of ATP and was reversed only by re-energization of the membranes followed by the immediate addition of ATP (table 1). Thus, binding of ADP in the dark transforms the activated enzyme to an inactive form which does not release or exchange the tightly bound ADP.

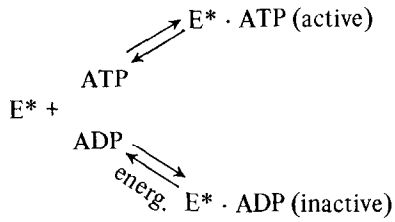
These results can be explained by the following events: the rapid release of tightly bound ADP appears to preceed the relatively slower process of energy-dependent conformational changes in the structure of the ATPase, that yields the fully active state. The overall rate of the activation process is rather slow with a $t_{1/2}$ of several minutes [3,19].

$$E \cdot ADP \underset{ADP}{\overset{\text{energ. ADP}}{\rightleftharpoons}} E \overset{\text{energ. DTT}}{\rightleftharpoons} E^* \qquad (1)$$

(energ. = energization)

The activated state of the ATPase [E*] decays rather slowly to an inactive form in the dark and in the absence of ATP ([3,7,19], fig.3a). The active conformation binds ATP or ADP in the dark (fig.1). Binding

of ATP gives a catalytically active [E* · ATP] complex while binding of ADP results in a catalytically inactive complex [E* · ADP].



(2)

The inactive [E* · ADP form can be reactivated by energization with the concomitant release of ADP. The reactivation of the inhibited enzyme is energy-dependent as is the initial activation but occurs considerably faster. It seems that reactivation depends mainly on the rapid release of tightly bound ADP while activation of the latent enzyme is a more complicated process. Our results do not exclude rapid conformational changes which may occur during the reactivation process. Activation of the latent ATPase correlates with the release of ADP [5]. Under the experimental conditions of [5] the activation process was complete after 5 s preillumination while in our experiments full activation was obtained after several minutes. However, based on the values for ATP hydrolysis [5] it is most probable that maximal ATPase activity was not reached due to the complete hydrolysis of the ATP and lack of substrate during a major part of the dark incubation period. Relatively low Mg^{2+}-ATPase activities were also detected by activation with short light flashes [4]. Thylakoid membranes probably contain a heterogenous population of ATPase molecules, some of them in the E* · ADP conformation, that can rapidly be transformed to the active form by release of ADP.

The inhibition of the P_i–ATP exchange and ATP hydrolysis by ADP was initially reported in [7] with the suggestion that the mobility of ADP across the thylakoid membrane is the cause for inhibition. We propose that binding of nucleotides to the tight-binding site(s) of the ATP synthetase controls the energy-dependent interconversion of inactive to active enzyme conformations. The active conformation of the ATP synthetase may be stabilized by the binding of ATP, while binding of ADP to the tight binding site(s) inactivates the enzyme. ATP tightly bound to the energized state of the ATP synthetase has been reported [12,20]. Thus the active enzyme conformation that participates in ATP synthesis, hydrolysis and the P_i ATP exchange reactions may contain tightly bound ATP. Upon turning the light off and if the concentration of ATP is low, the enzyme will bind ADP to form the de-energized inactive form (fig.3b). From the data given in fig.3b, the initial rate of ATP hydrolysis was ~20 mol . mol CF_1^{-1} . s^{-1}. It seems therefore that hydrolysis does not occur at the tight nucleotide-binding site but at the catalytic site where rapid binding and release can sustain such rates of hydrolysis. The ADP formed then binds to the tight site with inactivation of the enzyme. These aspects are being investigated further.

Acknowledgements

We wish to thank Mrs Z. Conrad and D. Rabinovitz for excellent technical assistance. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

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