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Interaction of Pyrophosphate with Catalytic and Noncatalytic Sites of Chloroplast ATP Synthase

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Abstract—The effect of pyrophosphate (PP_i) on labeled nucleotide incorporation into noncatalytic sites of chloroplast ATP synthase was studied. In illuminated thylakoid membranes, PP_i competed with nucleotides for binding to noncatalytic sites. In the dark, PP_i was capable of tight binding to noncatalytic sites previously vacated by endogenous nucleotides, thereby preventing their subsequent interaction with ADP and ATP. The effect of PP_i on ATP hydrolysis kinetics was also elucidated. In the dark at micromolar ATP concentrations, PP_i inhibited ATPase activity of ATP synthase. Addition of PP_i to the reaction mixture at the step of preliminary illumination inhibited high initial activity of the enzyme, but stimulated its activity during prolonged incubation. These results indicate that the stimulating effect of PP_i light preincubation with thylakoid membranes on ATPase activity is caused by its binding to ATP synthase noncatalytic sites. The inhibition of ATP synthase results from competition between PP_i and ATP for binding to catalytic sites.

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ATP synthase of chloroplast thylakoid membranes (CF_0CF_1) catalyzes photosynthetic phosphorylation generating ATP using light-induced transmembrane difference of proton electrochemical potentials ($\Delta\mu_{H^+}$). Similar to mitochondrial and bacterial ATP synthases, it consists of a peripheral water-soluble component (CF_1) and a membrane component (CF_0). The chloroplast coupling factor CF_1 comprises single γ-, δ-, and ε- subunits, as well as α- and β-subunits in three copies each. At the interfaces between the latter, there are three catalytic and three noncatalytic nucleotide binding sites [1, 2]. In the dark, ATP synthase is inactive. After preillumination in the presence of thiols, ATP synthase becomes capable of catalyzing dark ATP hydrolysis coupled with transmembrane proton transfer into the thylakoid [3]. Its activation is accompanied by release of ADP previously bound tightly to a catalytic site [4, 5]. Stability of its active state is low and affected by light cessation or uncoupler addition resulting in a decrease of proton transmembrane gradient. ATP synthase inactivation is accompanied by ADP binding [6, 7]. In the absence of an energy source, isolated coupling factors of various origin display only ATPase activity. Similarly, their activation/inactivation is accompanied by ADP dissociation/association [8-10], with the only difference consisting in high ATP concentrations or oxyanions as activation-inducing agents [10-13].

It was found at the beginning of 1990s that a prerequisite to activation of ATPase properties of coupling factors from chloroplasts, mitochondria, and bacteria is ATP binding to noncatalytic sites [14, 15], which promotes dissociation of ADP previously bound tightly to a catalytic site [16-18]. Also, there is evidence for interaction between oxyanions in the same role and noncatalytic sites [19]. But far less is known about the role of noncatalytic sites in ATP synthase functioning. To a certain extent, this results from the absence, until recently, of a technique for quantitative estimation of nucleotide binding to noncatalytic sites, and from a striking difference between nucleotide binding properties of ATP synthases, as well as peculiarities of ATP synthesis/hydrolysis, and those of F_1-ATPases. For example, inhibition of ATP hydrolysis by ATP synthase of chloroplast thylakoid membranes requires, as compared, a ten-fold concentration of MgADP [20]. Also, sodium azide, known as a stabilizer of the F_1-ATPase inactive state, produces virtually no effect on ATP synthase-cat-

Abbreviations: CF_0CF_1, chloroplast ATP synthase; DTT, dithiothreitol; PMS, phenazine methosulfate; TCA, trichloroacetic acid.

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alyzed oxidative and photosynthetic phosphorylation [21, 22]. Unlike CF_1-ATPase noncatalytic sites, those of chloroplast ATP synthase differ much from one another as to their nucleotide binding properties [23].

On the other hand, some similarity has been found in the effect produced by ATP and by oxyanions on the soluble and membrane-bound enzyme. As shown, sulfite anion, a most efficient F_1-ATPase stimulating oxyanion, activates chloroplast ATP synthase [24]. Light preincubation of thylakoid membranes in the presence of ATP stimulates their ATPase activity in the subsequent dark phase [25]. Phosphate and PP_i anions stabilize light-induced ATPase activity in the dark [6, 26]. Studies on the interaction of PP_i with isolated coupling factors of various origin revealed a number of its peculiarities fit for investigating the mechanism of regulation of ATP synthase activity. Structurally, phosphodiester-bonded PP_i is closer to ADP and ATP than to inorganic phosphate. The structural similarity is also supported by similar energies of ATP and PP_i hydrolysis [27]. Unlike other oxyanions, PP_i is similar to nucleotides as to tight binding to F_1-ATPases [28-33]. Interestingly, some authors present evidence for an interaction of PP_i with catalytic sites [32], while those describing mitochondrial and bacterial enzymes report on highly selective interaction of PP_i with noncatalytic sites [34, 35]. Lastly, according to [36], azide-derived labeled pyrophosphate has been proved to bind to amino acid residues pertaining to sites of the both types. Since PP_i is localized in the chloroplast stroma, it can effect activity of ATP synthase *in vivo* [37].

The goal of this paper was to study the interaction of PP_i with nucleotide binding sites of thylakoid membrane ATP synthase and to elucidate possible involvement of noncatalytic sites in stabilizing its ATPase activity.

MATERIALS AND METHODS

Chloroplast thylakoid membranes were isolated from pea leaves as described by Semenova et al. [38] with a modification described earlier [39]. Chlorophyll concentration was determined according to Arnon [40]. Thylakoid membranes were light activated with 560 W/m^2 photosynthetically active radiation for 5 min. To prevent heating of the reaction mixture, an SZS-27 filter was used. The activation was performed in the mixture containing 20 mM Tricine-KOH (pH 7.8), 0.2 M sucrose, 20 mM KCl, 1 mM $MgCl_2$ (TSKM-buffer), 50 μM phenazine methosulfate (PMS), 10 mM dithiothreitol (DTT), and 0.2 mg/ml chlorophyll. To remove endogenous nucleotides, the medium was diluted with 5-10 volumes of TSKM-buffer 0.5 min before the preincubation was over. The membranes were precipitated by centrifugation and resuspended to give a final chlorophyll concentration of 1-2 mg/ml. Nucleotide binding to thylakoid membrane ATP synthase was performed at room temperature in the dark or in white light (560 W/m^2) in 0.7-1.4 ml medium containing [α-^{32}P]ATP, TSKM buffer, 50 μM PMS, and 0.1-0.2 mg/ml chlorophyll. The added pyrophosphate was complexed with magnesium. The labeled nucleotides were removed from catalytic sites using a modification of the previously described "cold chase" technique [39] based on enzyme-catalyzed substitution of unlabeled nucleotides for labeled ones. The reaction medium was supplemented with solution (10% of its volume) containing 22 mM ATP, 0.66 M $KHSO_3$ (pH 7.8), 50 μM diadenosine pentaphosphate, and 50 μM gramicidin D. After 2 min, the mixture was centrifuged for 1 min at 10,000*g*. To remove free nucleotides, the post-centrifugation pellet was washed three times and resuspended in 90 μl buffer containing 0.2 M sucrose, 50 mM Tricine-KOH (pH 7.8), 50 mM KCl, and 5 mM $MgCl_2$. For chlorophyll determination, 20-40 μl of the resultant suspension was utilized, and the rest was used for nucleotide extraction by 0.5 M $HClO_4$ followed by neutralization with KOH. Chromatographic separation of nucleotides from $HClO_4$ extracts was carried out by thin layer chromatography. The nucleotide content was counted from radioactivity of nucleotide spots.

The rate of dark hydrolysis was determined from [α-^{32}P]ATP and [α-^{32}P]ADP content in the reaction mixture.

Light-induced ATP hydrolysis was measured by potentiometric titration using a glass electrode [41]. The reaction was carried out in a thermostatted cell at 20°C in 5 ml reaction mixture containing 2 mM Tricine-KOH (pH 7.8), 0.2 M sucrose, 100 mM KCl, 2 mM $MgCl_2$, 0.05 mM PMS, and thylakoid membranes (20 μg chlorophyll/ml). Thylakoid membranes were reduced by DTT as described above with the exception of the endogenous nucleotide dissociation step. After 2 min thylakoid membrane activation with white light of 500 W/m^2 intensity, the reaction was initiated by addition of 0.5 mM ATP and 1 mM NH_4Cl. The added pyrophosphate was complexed with magnesium. High stability constant of this complex [42] ruled out the effect of these additions on buffer capacity and on concentration of free magnesium. Reaction details are given in figure legends.

RESULTS

The interaction of PP_i with noncatalytic sites was estimated from the decrease of tight binding of labeled ADP and ATP to these sites. This approach was used because, according to the literature [43], ATP synthase is the only chloroplast enzyme providing tight binding of nucleotides, whereas for PP_i no such a property has been reported. Prior to PP_i experiments, the noncatalytic sites of ATP synthase were vacated by endogenous nucleotides, as described under "Materials and Methods". Figure 1 (curve *1*) shows that in the dark 500 μM PP_i notably inhibits incorporation of labeled nucleotides into ATP

synthase noncatalytic sites. Under conditions of light incubation at low nucleotide concentrations the observed incorporation was very low (Fig. 1, curve *2*), and it ceased completely at high nucleotide concentrations (Fig. 1, curve *3*). Only millimolar concentrations of PP_i (Fig. 2) resulted in a considerable inhibition of nucleotide incorporation in the light. The PP_i concentration providing a half-maximal inhibition under these conditions was about 2.5 mM. The interaction of PP_i with noncatalytic sites in the dark was much higher, provided the thylakoid membranes had been incubated with PP_i prior to incorporation

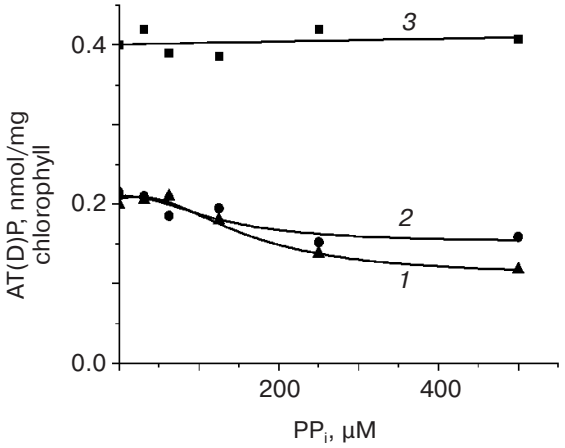

Fig. 1. Nucleotide incorporation into ATP synthase noncatalytic sites as dependent on PP_i concentration and preincubation conditions. Thylakoid membranes (0.1-0.2 mg chlorophyll/ml) were incubated with various amounts of PP_i in medium containing TSKM-buffer, 5 μM gramicidin (in the dark, curve *1*) or 50 μM PMS (in the light, curves *2* and *3*), and 5.5 (curves *1* and *2*) or 24.7 μM (curve *3*) [α-^{32}P]ATP for 5 min.

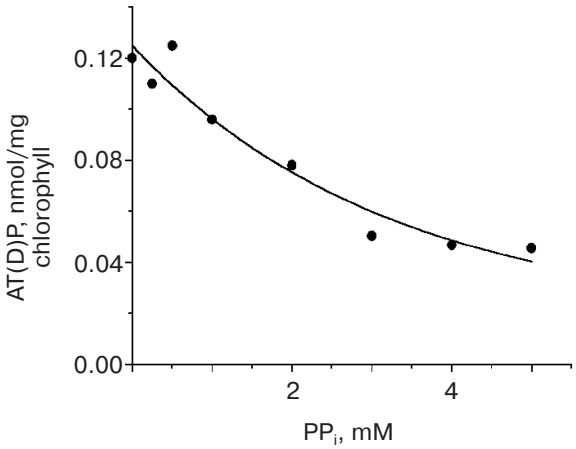

Fig. 2. Effect of high PP_i concentrations on nucleotide incorporation into ATP synthase noncatalytic sites. Thylakoid membranes (0.1-0.2 mg chlorophyll/ml) were light incubated with various amounts of PP_i in medium containing TSKM buffer, 50 μM PMS, and 0.7 μM [α-^{32}P]ATP for 7 min. For further procedures, see legend to Fig. 1 and "Materials and Methods".

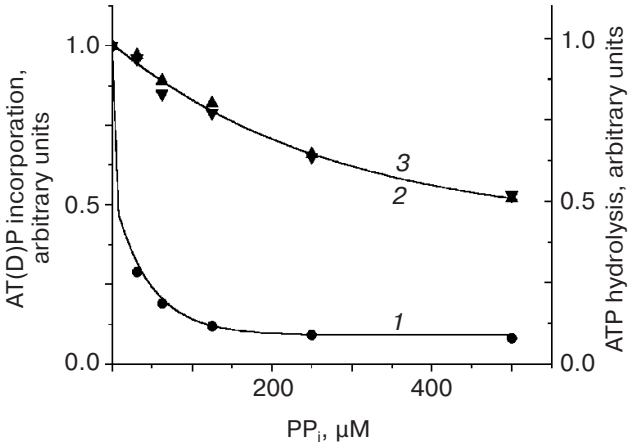

Fig. 3. Effect of thylakoid membrane preincubation with PP_i on nucleotide incorporation into noncatalytic sites and on apparent rate constants of ATP hydrolysis. Thylakoid membranes (0.22 mg chlorophyll/ml) were incubated for 5 min in medium containing TSKM buffer, 5 μM gramicidin, and 4.4 μM [α-^{32}P]ATP. PP_i was added either 3 min before [α-^{32}P]ATP addition (curves *1* and *3*) or together with it (curve *2*). Incorporation of 0.20 nmol AT(D)P per mg chlorophyll (curve *1*) and the first order apparent rate constant of 0.19 min^{-1} (curve *2* and *3*) were taken as unity. For further procedures, see legend to Fig. 1 and "Materials and Methods".

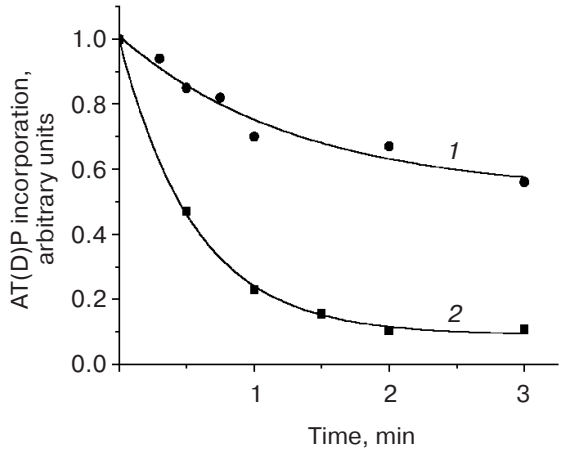

Fig. 4. Nucleotide incorporation into noncatalytic sites as dependent on time of preincubation of thylakoid membranes with pyrophosphate. Thylakoid membranes (0.26 mg chlorophyll/ml) were incubated for 3 min with 31 (*1*) or 125 μM PP_i (*2*); then 4.0 μM [α-^{32}P]ATP was added, and the incubation was continued for 5 min more. Incorporation of 0.15 nmol AT(D)P per mg chlorophyll was taken as unity. The extent of nucleotide binding to noncatalytic sites was determined as described under "Materials and Methods".

of labeled nucleotides (Fig. 3, curve *1*). Preincubation for 5 min allowed half-maximal effect at a PP_i concentration of about 25 μM. The rate of PP_i binding increased with its increasing concentration (Fig. 4). However, even with 125 μM PP_i this rate was an order of magnitude lower than

the value we found previously for the nucleotide binding rate, whereas the concentration providing half-maximal binding was an order of magnitude higher [39]. This dependence of nucleotide incorporation on preincubation of thylakoid membranes with PP_i indicates that in the dark PP_i can tightly bind to vacant noncatalytic sites of ATP synthase.

To learn how PP_i binding to noncatalytic sites influences the functional activity of ATP synthase, kinetics of dark- and light-induced ATP hydrolysis was studied. In the dark, DTT-reduced ATP synthase of chloroplasts is known to exhibit minor ATPase activity [3]. ATP labeling allowed studying kinetics of ATP hydrolysis at micromolar nucleotide concentrations, with the stimulating effect of oxyanions either absent or negligible [44]. As seen in Fig. 3 (curve *3*), increased PP_i concentrations caused a notably decreased effective constant of the reaction rate. The half-maximal inhibition was achieved at ~0.5 mM PP_i. Importantly, filling of noncatalytic sites as a result of preincubation with PP_i produced virtually no effect on the hydrolysis rate effective constant, as seen in Fig. 3 where curves *2* and *3* (without and with preincubation) nearly coincide. Thus, inhibition of ATP hydrolysis does not correlate with pyrophosphate binding to noncatalytic sites of ATP synthase.

A short light incubation of thylakoid membranes activated ATP synthase, namely, immediately after light cessation and addition of uncoupler and ATP, it provided rapid hydrolysis, the rate of which gradually decreased with decreasing enzyme activity (Fig. 5, curve *1*). The presence of PP_i during light incubation slightly decreased the reaction rate at the early stage and hampered inactivation of the enzyme (Fig. 5, curve *2*). The same rate decrease resulted from PP_i addition at the dark stage, although the inactivation process remained almost unaffected (Fig. 5, curve *3*). Figure 6 presents initial hydrolysis rates and effective constants of enzyme inactivation versus PP_i concentration, as calculated from the first-order equation. With PP_i concentration increased to 1 mM, a twofold decrease of the inactivation constant occurs, while the initial reaction velocity decreases slightly.

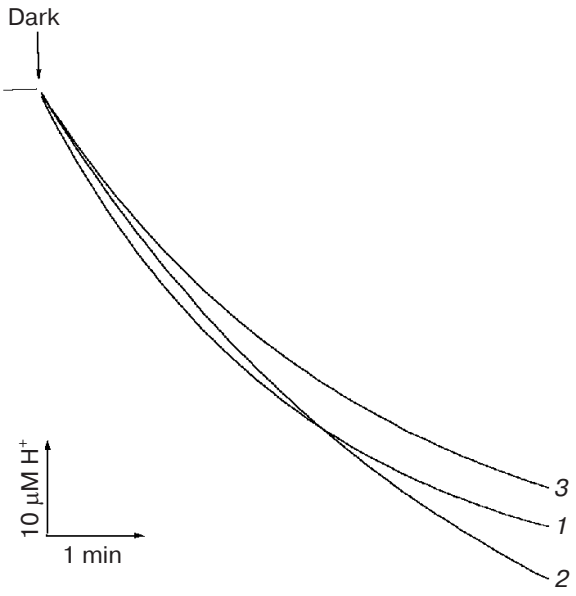

Fig. 5. Effect of PP_i on kinetics of light-induced ATP hydrolysis. Thylakoid membranes (0.02 mg chlorophyll/ml) were incubated in the light for 2 min in the absence (curves *1* and *3*) or the presence (curve *2*) of 2.5 mM PP_i. Immediately after light cessation, ATP (0.5 mM), NH_4Cl (1 mM), and PP_i (2.5 mM) (curve *3*) were added to the reaction medium (5 ml) that contained 2 mM Tricine-KOH (pH 7.8), 0.2 M sucrose, 0.1 M KCl, 2 mM $MgCl_2$, and 0.05 mM PMS.

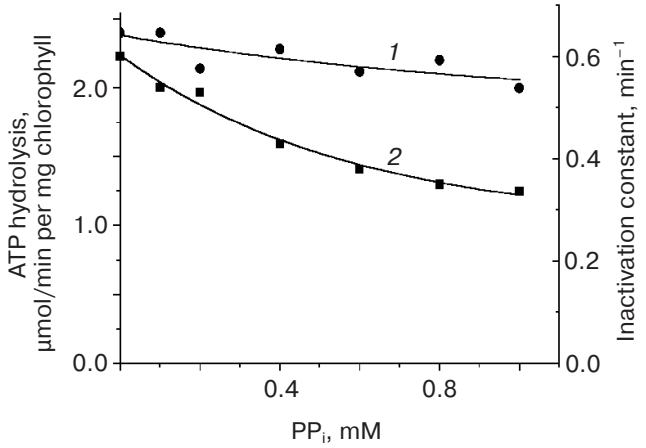

Fig. 6. Initial rate (*1*) and inactivation constant (*2*) of light-induced ATP hydrolysis as dependent on PP_i concentration. For reaction conditions, see legend to Fig. 5. The initial rate was determined 40 sec after addition of ATP and NH_4Cl.

DISCUSSION

The results presented above show that, depending on experimental conditions, PP_i can produce either inhibiting or simulating effect on ATPase activity of ATP synthase. During light-induced hydrolysis at a high ATP concentration, the inhibition occurred immediately after light cessation, with the enzyme still active. It was also recorded in the dark under conditions of a quasi-stable state at ATP concentrations much lower than hydrolysis K_m (90 μM [6]). Importantly, the inhibition did not correlate with PP_i binding to noncatalytic sites of ATP synthase (Fig. 3, curves *1-3*). With structural similarity between PP_i and the ATP polyphosphate group taken into account, this can be supposed to result from competition for binding to catalytic sites. Thus, pyrophosphate (rather, its combination with magnesium) is capable of interacting with both catalytic and noncatalytic sites of

chloroplast ATP synthase. Its interaction with catalytic sites does not require any preliminary light incubation of thylakoid membranes and results in inhibition of ATP hydrolysis (Fig. 3). The extent of inhibition decreases with increasing ATP concentration (cf. Figs. 3 and 6), which is in agreement with the expected competitive mechanism of inhibition. The interaction of pyrophosphate with noncatalytic sites demands no energization of the thylakoid membrane as well, provided endogenous nucleotides have vacated these sites (see "Materials and Methods"). Depending on PP_i concentration, its tight binding to noncatalytic sites takes a period from a few seconds to several minutes (Figs. 3 (curve *1*) and 4). In the light, due to low PP_i affinity for noncatalytic sites under conditions of thylakoid membrane energization, considerable binding of PP_i occurs only at its high concentrations (Fig. 2).

As follows from comparison between the interaction of PP_i with noncatalytic sites and its effect on inactivation of ATP synthase, the main parameters of these two events are quite close. Indeed, unlike inhibition resulting from PP_i-catalytic site interaction, decreasing inactivation (stimulation) of ATPase activity requires the presence of PP_i during light preincubation of the membrane. The necessity of preincubation is connected with the low rate of PP_i binding to noncatalytic sites that release endogenous nucleotides in the course of thylakoid membrane energization (in this set of experiments, the step of preliminary dissociation of endogenous nucleotides was omitted). Addition of PP_i, along with ATP, after light cessation produced no notable stimulating effect (Fig. 5, curve *3*). Activity-stimulating PP_i concentrations appeared to be much higher than those providing efficient PP_i binding to vacant noncatalytic sites in the dark, but close to those required for this purpose in the light. The conclusion on participation of noncatalytic sites in stimulation of ATP activity of chloroplast ATP synthase is in agreement with the previous suggestion that ATPase activity of F_1-ATPases can be stimulated by the interaction of ATP/oxyanion with their noncatalytic sites [14, 15, 19, 45, 46]. According to [19], the activating effect of oxyanions can result from their replacing ATP γ-phosphate near bound ADP, whereas PP_i, that interacts with a noncatalytic site using both its phosphate groups can perform binding solely on condition of nucleotide dissociation. This also explains why PP_i is capable of tight binding to a noncatalytic site under conditions of membrane deenergization.

Previously, the literature and our experimental results suggested that the effect of oxyanion binding to noncatalytic sites on the enzyme activity is realized through weakening ADP interaction with one of the catalytic sites [19]. Then, a question arises as to the mode of signal transduction from an α-subunit noncatalytic site to a catalytic site of the β-subunit. Sokolov and colleagues reported [47] that γ-subunit-free CF_1 complexes lose the ability of being stimulated by sulfite. Later, Richter's and McCarty' teams showed that mutations occurring at a certain site of interaction between the γ-subunit and the α- and β-subunits also result in the loss of sulfite-induced stimulation of the enzyme [48, 49]. These results indicate that stimulation of ATPase activity by oxyanions and specifically by PP_i is caused by their interaction with noncatalytic sites followed by transfer of these interaction-induced conformational changes to the ADP tightly binding catalytic site via the γ-subunit.

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