THE REQUIREMENTS OF ADENOSINE DIPHOSPHATE FOR LIGHT-TRIGGERED ATPase AND ATP-Pi EXCHANGE REACTIONS IN CHLOROPLASTS

C. CARMELI and Y. LIFSCHITZ
Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel

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1. Introduction

The requirement for ADP and Pi in exchange reactions in chloroplasts was assumed to indicate that the reactions leading to ATP synthesis do not involve a covalently bound enzyme intermediate [1]. It is known, however, that the chloroplast membrane is rather impermeble to various anions and cations [2]. Therefore, the reactants concentration at the catalytic site is not necessarily reflected by their concentration in the outside medium. In this work a disparity between the internal and external concentrations of ADP and Pi was indicated by the effect which the addition and the removal of these reagents had on light-triggered ATPase and on ATP-Pi exchange reactions in chloroplasts. The requirements for ADP and Pi in light-triggered ATP-Pi exchange reaction indicated that the exchange was a result of a dynamic reversal of photophosphorylation.

2. Materials and methods

Once washed chloroplasts were prepared from lettuce, as previously described [3]. ATP-Pi exchange and ATPase reactions were assayed at 22° in a reaction mixture containing: Tricine – NaOH, pH 8, 30 mM; KCl, 50 mM; $MgCl_2$, 10 mM; Na, K phosphate, pH 8, 0.25 mM (containing 10^6 counts of ^{32}P/min); ATP, 5 mM; DTT, 5 mM; FMN, 0.1 mM, and chloroplasts suspension containing 50 μg chlorophyll in a total volume of 1 ml. When indicated PEP (phospho enoyl pyruvate), at a final concentration of 2 mM, and PK were added to the reaction mixture. PK (pyruvate kinase) from rabbit muscle, A grade, was purchased from Calbiochem and was prepared by dialysis of 0.2 ml of the enzyme against 2 l of Tris-HCl, pH 8, 5 mM and KCl, 2 M for 24 hr at 0–4°. PK activity was assayed according to the method of Bucher [4]. Routinely, the reaction mixture was illuminated for 5 min at 70,000 lux in the absence of ATP and PEP. ATP and PEP (when indicated) were added immediately after light was turned off and the reaction was allowed to proceed in the dark for 10 min. It was terminated by the addition of trichloracetic acid to a final concentration of 3%. ATP-Pi exchange reaction was assayed by the determination of [^{32}P] ATP content according to the isobutanol-benzene extraction procedure [3]. ATPase activity was assayed by the determination of Pi content according to the method of Ames [5], and chlorophyll content was determined according to the method of Arnon [6].

3. Results and discussion

The effect of various ADP concentrations on light-triggered ATPase and ATP-Pi exchange reactions is shown in fig. 1. At higher ADP concentrations both reactions are inhibited. As was previously shown both light-triggered ATPase [7] and ATP-Pi exchange [8] reactions are competitively inhibited by ADP. The linearity of the time course of light-triggered ATPase activity (fig. 2) excludes the possibility of inhibition by the ADP produced during the reaction. Product inhibition would have caused a decrease in the rate of activity with time. Calculation from fig. 2 shows that after 10 min of the reaction ADP concentration increased to 0.4 mM. According to the results shown

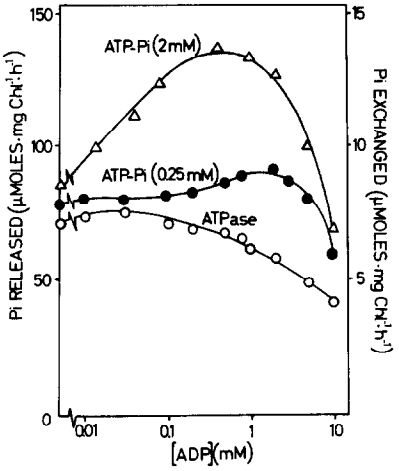

Fig. 1. The effect of ADP on ATPase and ADP-Pi exchange activities.

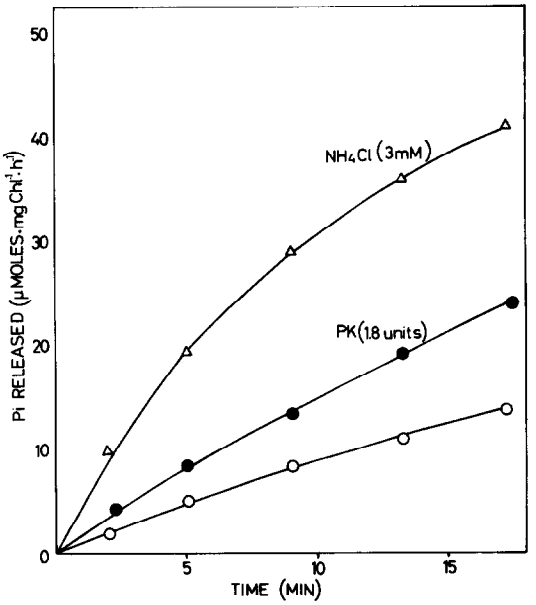

Fig. 2. The effect of NH_4Cl and PK on the time course of ATPase activity.

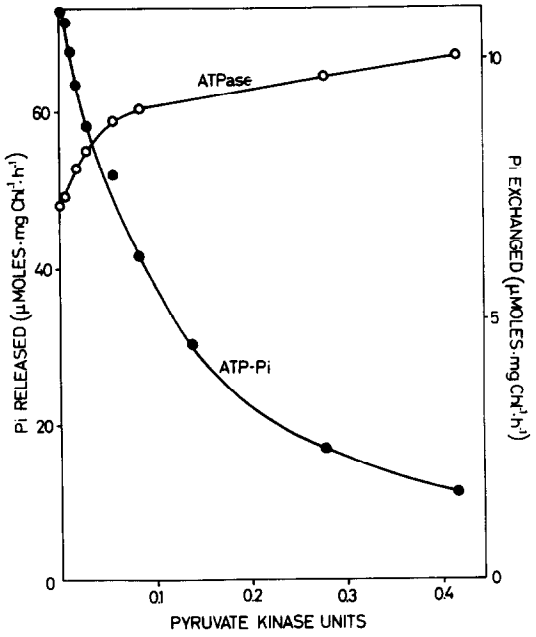

Fig. 3. The effect of PK on ATPase and ATP-Pi exchange activities.

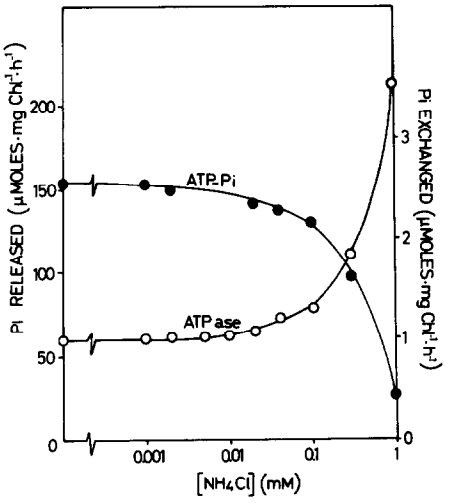

Fig. 4. The effect of NH_4Cl on ATPase and ATP-Pi exchange activities.

Table 1
The effect of PK on ATPase activity

Additions	Rate of ATPase activity (μ moles Pi X mg Chl^{-1} X hr^{-1})
none	33.6
PK 3 units, PEP	66.3
PK 3 units	33.8
PEP	32.4
PEP, -ATP	0
PK 3 units, PEP, -ATP	0

in fig. 1, initially added 0.4 mM ADP gave only 2% inhibition of the activity. Another test for possible product inhibition involves the removal of the product during the course of the reaction. ADP can be rephosphorylated to ATP by PK and PEP. The addition of PK and PEP doubled the rate of ATPase activity (table 1). The increase in the rate of ATPase activity was not caused by PK itself or by any possible impurities added with it. Neither did PEP by itself stimulate ATPase activity. It can also be seen from table 1 that in the absence of ATP there was no light-triggered PEP hydrolysis, whether PK was added or not. Uncouplers were shown to stimulate ATPase activity [9]. However, there are several indications that PK does not act as an uncoupler. The time course of ATPase activity stimulated by PK was linear while the rate of ATPase activity stimulated by the uncoupler NH_4Cl decreased with time (fig. 2). Other uncouplers have a similar effect on the rate of ATPase activity [9]. In unpublished experiments it was shown that electron transport in chloroplasts from H_2O to ferricyanide or to O_2, which was stimulated by the uncoupler NH_4Cl, was not stimulated by PK plus PEP.

Increasing PK concentrations caused an increase in ATPase activity (fig. 3). The first 0.035 PK units stimulated ATPase activity from 0.04 to 0.046 units, an increase of 15%. Doubling of the PK units only caused an additional 7% increase in ATPase activity. The data indicates that when ATPase activity became rate limiting the additional stimulation of ATPase activity per PK unit decreased. It can be assumed that PK remains outside while the ATPase is located inside the chloroplast. Rephosphorylation of ADP by PK reduced the steady state level of ADP outside the chloroplast. It was shown above that the ADP which was produced during ATPase activity was not sufficient to cause inhibition of the activity if added to the medium. Therefore, the removal of these small concentrations of ADP by PK could not have caused a stimulation of ATPase activity unless it is assumed that ADP concentration inside the chloroplast is higher than in the medium. It seems that the movement of the ADP produced during ATPase activity to the outside is barred by the membrane. PK activity lowered the steady state concentration of ADP in the medium. The increased gradient of concentrations produced by PK caused a faster movement of ADP to the medium. A 100% stimulation caused by 3 units of PK would mean that the ADP which was removed by the PK caused a 50% inhibition of ATPase activity. Since an addition of 7 mM caused 50% inhibition of ATPase activity, it can be speculated that the internal concentration of ADP during ATPase activity was of that order of magnitude. The effect of PK on ATPase activity could not have resulted from higher concentrations of ATP which was produced by PK, since the decrease in ATP during the course of the reaction did not change the rate of ATPase activity as can be seen from the linearity of the time course.

PK decreased ATP-Pi exchange activity simultaneously with the stimulation of ATPase activity (fig. 3). At the same PK concentration the extent of inhibition of ATP-Pi exchange was greater than the extent of stimulation of ATPase activity. The stoichiometry, however, shows a smaller decrease in the rate of ATP-Pi exchange than in the rate of ATPase activity. The stimulation of ATPase activity by PK was only partially due to the simultaneous inhibition of ATP-Pi exchange activity. The net effect of PK on ATPase activity could be obtained by substruction of the rate of ATP-Pi exchange which was inhibited from the total rate of ATPase activity at a given PK concentration. The removal of ADP by PK caused an inhibition of ATP-Pi exchange. The absolute requirement of ADP for ATP-Pi exchange indicated that the exchange was a result of a dynamic reversal of phosphorylation and not a result of the formation of a covalently bound enzyme intermediate. NH_4Cl, an uncoupler of phosphorylation also inhibited ATP-Pi exchange simultaneously with the stimulation of

ATPase activity (fig. 4). Unlike PK, the percentage of stimulation of ATPase by NH_4Cl was greater than the percentage of inhibition of ATP-Pi exchange caused by the uncoupler. Such difference in the effect of NH_4Cl and PK on the two reaction probably resulted from the difference in their mode of action.

The inhibition of ATP-Pi exchange activity by PK could also be the result of a decrease in the concentration of ADP available for rephosphorylation of ADP with Pi. If this were the case, the concentration of ADP during ATP-Pi exchange was not necessarily optimal for the activity. Indeed addition of ADP caused a stimulation of ATP-Pi exchange at concentrations of ADP which already inhibited ATPase activity (fig. 2). From fig. 2 it can be seen that the stimulation of ATP-Pi exchange activity by ADP was also dependent on Pi concentration. A lower ADP concentration was required for the stimulation of ATP-Pi exchange at 2 mM than at 0.25 mM Pi. The extent of stimulation of ATP-Pi exchange by ADP was also greater at higher Pi concentrations. However, the same ADP concentrations inhibited ATP-Pi exchange activity whether the Pi concentration was high or low. The high requirements for Pi in ATP-Pi exchange activity could indicate that Pi penetration through the chloroplast membrane is slow. As was suggested [10] ADP aids phosphate penetration across the membrane. The stimulation of ATP-Pi exchange activity by ADP could be the result of both an increase in ADP concentration at the catalytic site and a faster rate of Pi penetration. Since the incorporation ^{32}Pi into ATP served as a measurement for ADP-Pi exchange activity, the apparent rate would be slower than the actual rate of the exchange, if ^{32}Pi penetration through the membrane was rate limiting.

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