ON THE ROLE OF PHOSPHORYLATED INTERMEDIATES IN SODIUM AND POTASSIUM STIMULATED ATP-HYDROLYSIS*

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Received March 15, 1966

The transport of Na^+ and K^+ against an electrochemical gradient is supposed to require ATP as an energy source (1). A Na^+ and K^+ stimulated ATP-ase has been shown to be related with this process (2). To elucidate the mechanism of Na^+ and K^+ transport, the mechanism of Na^+ and K^+ stimulated ATP hydrolysis was studied with purified enzyme preparations. The exchange of ^{32}P between ADP^{32} and ATP and observations with terminally labeled ATP^{32} (2,3,4,5,6,7) are consistent with a mechanism which involves a Mg^{++}, Na^+ dependent phosphorylation of an acceptor group of the enzyme complex (equ. 1) and a subsequent K^+ dependent hydrolysis of this intermediate (equ. 2)(3,4,5,6,7).

$$(1) \quad ATP + E \xrightleftharpoons{Mg^{++},\ Na^+} E\sim P + ADP$$

$$(2) \quad E\sim P + H_2O \xrightarrow{K^+} E + P$$

The phosphorylated intermediate could be shown to be stable against acid (6,7), whereas alkalinity (4,6,7) or hydroxylamine (6,7) liberate phosphate. The latter finding points to an acylphosphate to be an intermediate in Na^+ and K^+ stimulated ATP hydrolysis (6,7).

This paper concerns the effects of hydroxylamine, trinucleotides (ATP, GTP, ITP) and dinucleotides (ADP, GDP, IDP) on the labeling of purified ATP-ase preparations by ATP^{32} and the effect of hydroxylamine and dinucleotides on the rate of ATP hydrolysis. From the differences

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* A summary of these investigations has been presented at the meeting of the Gesellschaft für physiologische Chemie, Berlin, October 1965 (8).

in the behaviour of the various nucleotides as substrates in each system and from the inability of hydroxylamine to inhibit ATP-hydrolysis it is concluded that the acid stable phosphorylated product is not involved in Na^+ and K^+ stimulated ATP hydrolysis.

MATERIAL AND METHODS

Preparation of ATP-ase: The Na^+, K^+ stimulated ATP-ase preparations were isolated by use of an coupled optical assay with pyruvate kinase and lactic dehydrogenase from ox brain in a particulated form by incubation (5 to 7 days at 0° C) of crude preparations with desoxycholic acid, subsequent fractionation in the ultracentrifuge, chromatography on Bio-Gel 100 and repeated differential centrifugation[1]. The specific activities obtained by this procedure were 1.7 to 3.0 units / mg protein (1 unit being the amount of enzyme catalyzing the Mg^{++}, K^+ and Na^+ dependent hydrolysis of 1 µmole ATP / min. at 37° C). The ratios of activities $[(Mg^{++} + K^+ + Na^+) - Mg^{++}]/ Mg^{++}$ varied from 40 to 60. The enzyme preparation was free of succinic dehydrogenase, palmityl-CoA deacylase (9), reducing enzyme (10) and cytochrome c reductase (11).

Preparation of ATP^{32}: Terminally labeled ATP^{32} with a specific radioactivity of 16.3×10^6 cpm / umole was prepared and purified according to Glynn and Chappell (12) with the following modification[2]: after removal of ADP from the Dowex 1 column ATP^{32} was eluted with 250 ml of a mixture containing 0.01 n HCl and 0.2 M LiCl, neutralized with LiOH and lyophilisized. The residue was dissolved in 4 - 5 ml of water and ATP precipitated as the barium salt by the addition of 0.2 ml saturated $BaBr_2$ solution. The precipitate was decomposed with Dowex 50 (H^+-form). The supernatant was neutralized with 2-amino-2-methyl-1,3-propandiol (AMPD) (2).

Preparation of the acid stable phosphorylated intermediate: ATP-ase (5 - 7 mg protein / ml) was washed 3 times with 3 volumes 0.25 M sucrose to remove traces of K^+ and Na^+ and resuspended in half of the volume of 0.25 M sucrose.

To 1 ml containing 100 µmoles imidazole buffer pH 7.5, 5µmoles Mg^{++}, 0.1 µmoles ATP^{32}, 0.3 mg of ATP-ase protein were added. Both the reaction mixture and the enzyme were preincubated for 2 minutes at 30° C. After

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1) W. Schoner, Chr. v. Ilberg, R. Kramer and W. Seubert: in preparation.
2) We thank Dr. G. Hartman for these suggestions.

0 - 50 seconds the reaction was stopped by the addition of 4 ml 4% $HClO_4$ (0° C). The precipitate was isolated by filtration through millipore filter (Millipore Filter Corporation, Bedford, Mass., 0.8 μ pore size) and washed 3 times with 0.5 n $HClO_4$ (10^{-4} M ATP and inorganic phosphate). The dried filters were assayed in the Tricarb with PPO/POPOP.

Assay of ATP-ase in the presence of NH_2OH, ADP, GDP and IDP: After incubation of the enzyme at 37° C the reaction was stopped by the addition of 1 ml 4% $HClO_4$. After neutralization with K_2CO_3 the residual ATP was determined according to Lamprecht (13). $NH_2OH \cdot HCl$ was neutralized with Dowex 1 (OH^--form).

RESULTS AND DISCUSSION

In accordance with other investigators (1,3,4,5,6,7) incubating our ATP-ase with ATP^{32} led to successively increasing labeling of the particles in the presence of Mg^{++} and $Mg^{++} + Na^+$, respectively (Fig. 1). The label disappeared upon incubation with tri- and dinucleotides (Fig. 1a and 1b, equ. 3).

$$(3)\quad E\sim P^{32} + ADP\ (GDP,\ IDP) \xrightleftharpoons{Mg^{++},\ Na^+} E + ATP^{32}\ (ATP,\ GTP,\ ITP)$$

Most likely to this is due to an exchange of the enzyme bound phosphate with the terminal phosphate group of the trinucleotides and to a shift of the equilibrium of the equation 3 towards the right, respectively (ratio ATP^{32} / dinucleotide = 1 / 50). These findings suggest a broad specificity of the acceptor group towards purine nucleotides.

Hokin et al. (7) reported that digestion of the labeled enzyme preparations by pepsin liberated radioactive peptides which migrated towards the cathode at acid pH. Incubation with hydroxylamine or with acyl phosphatase liberated most of the label as inorganic phosphate. This result, which has been confirmed by this laboratory (Fig. 2), suggests an acyl phosphate to be the energy rich intermediate in the Na^+ and K^+ stimulated ATP hydrolysis (equ. 4 and 5)(7).

$$(4)\quad E\text{-}COOH + ATP \xrightleftharpoons{Mg^{++},\ Na^+} E\text{-}C(=O)\text{-}OP + ADP$$

$$(5)\quad E\text{-}C(=O)\text{-}OP + H_2O \xrightarrow{K^+} E\text{-}COOH + P$$

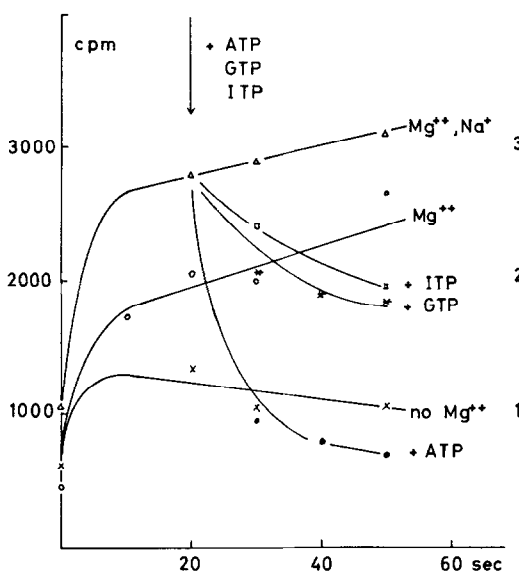

Fig. 1a: Effect of ATP, GTP and ITP on the Na^+-dependent incorporation of ^{32}P into ATP-ase. Na^+ 150 mM, at the time indicated 5 µmoles Mg-ATP (Mg-GTP, Mg-ITP) were added in 0.1 - 0.13 ml (for details see methods).

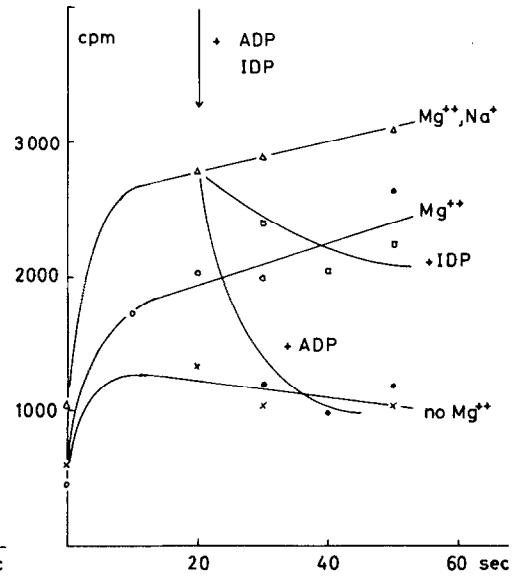

Fig. 1b: Effect of ADP and IDP on the Na^+-dependent incorporation of ^{32}P into ATP-ase. Na^+ 150 mM, at the time indicated 5 µmoles Mg-ADP or Mg-IDP were added in 0.12 ml (for details see methods).

We tested this possibility by investigating the effect of hydroxylamine on the overall hydrolysis of ATP, because hydroxylamine by blocking the acceptor group should irreversibly inactivate the ATP-ase (equ. 4 and 6).

(6) $E\text{-}C\overset{O}{\diagdown}_{OP} + NH_2OH \longrightarrow E\text{-}C\overset{O}{\diagdown}_{NHOH} + P$

(7) $E\text{-}C\overset{O}{\diagdown}_{NHOH} + H_2O \longrightarrow E\text{-}COOH + NH_2OH$

If, however, the enzyme bound hydroxamic acid were unstable (equ. 7), NH_2OH should substitute for K^+ in the overall reaction (equ. 5 = equ. 6 + 7). None of the postulates agree with the experimental data: Na^+ and K^+ stimulated ATP hydrolysis is not inhibited, even at concentrations of 0.8 M NH_2OH (Table I). Also in the presence of NH_2OH optimal rates of ATP hydrolysis depend on K^+ ions (not shown). These results exclude a

rôle of the enzyme bound acyl phosphate in the discussed reaction.

A participation of the phosphorylated intermediate is doubtful also in view of a study of the inhibitory effect of various dinucleotides

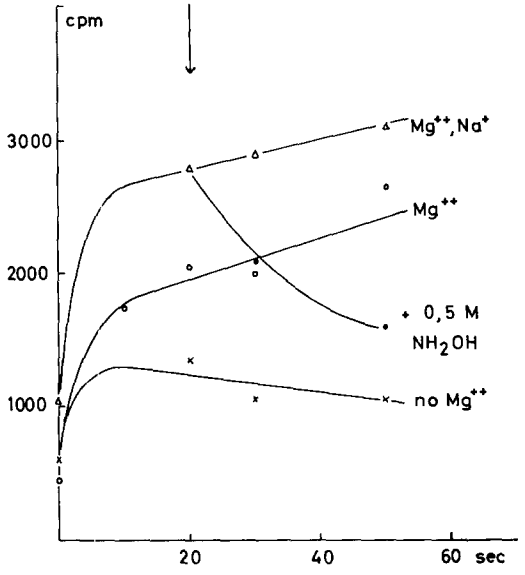

Fig. 2: Effect of NH_2OH on the Na^+-dependent incorporation of ^{32}P into ATP-ase. Na^+ 150 mM, 20 sec. after starting 1 mmole NH_2OH was added in 1 ml (for details see methods).

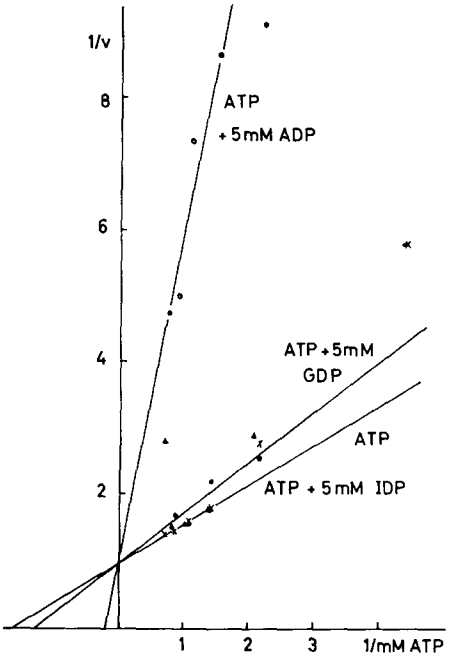

Fig. 3: Effect of dinucleotides on Na^+, K^+ stimulated ATP hydrolysis (Lineweaver - Burk reciprocal plots). 100 mM imidazole buffer pH 7.3, 3 mM Mg^{++}, 5 mM K^+, 100 mM Na^+. Incubation for 5 minutes.

on ATP hydrolysis. According to the isotope studies (Fig. 1a and b) ADP, IDP and GDP should act as competitive inhibitors of ATP-ase by shifting the equilibrium of the first step of the overall reaction toward left (equ. 1). As shown in Fig. 3, however, only ADP fulfills this postulate.

There may be a second phosphorylated intermediate participating in ATP hydrolysis, since ATP and ADP affect the labeling of ATP-ase by ATP^{32} more strongly than other nucleotides. These differences, however,

Table I

Influence of NH_2OH on ATP hydrolysis by Na^+, K^+ - ATP-ase

NH_2OH (M)	-Δumoles ATP / 15 min.
none	1.25
0.1	1.7
0.2	1.45
0.3	1.35
0.5	1.4
0.8	1.35

66 mM imidazole buffer pH 7.3, 3.3 mM $MgCl_2$, 3.3 mM KCl, 100 mM NaCl, 2 mM ATP.

may also be due to increased rates of the exchange and reverse reaction (equ. 3). A kinetic analysis of the effects of unlabeled ATP and GTP on the labeling over a longer period shall clarify this point.

This work was supported by grants from the National Science Foundation (NSF -6- 22 1o7, E. Heinz). The authors thank Prof. Heinz for his encouragement.

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