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Presence of a low-affinity nucleotide binding site on the $(K^+ + H^+)$-ATPase phosphoenzyme

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The effects of Mg^{2+} and nucleotides on the dephosphorylation process of the $(K^+ + H^+)$-ATPase phosphoenzyme have been studied. Phosphorylation with [γ-^{32}P]ATP is stopped either by addition of non-radioactive ATP or by complexing of Mg^{2+} with EDTA. The dephosphorylation process is slow and monoexponential when dephosphorylation is initiated with ATP. When phosphorylation is stopped by complexing of Mg^{2+} the dephosphorylation process is fast and biexponential. The discrepancy could be explained by a nucleotide mediated inhibition of the dephosphorylation process. The $I_{0.5}$ for ATP for this inhibition is 0.1 mM and that for ADP is 0.7 mM, suggesting that a low-affinity binding site is involved. When Mg^{2+} is present in millimolar concentrations in addition to the nucleotides the dephosphorylation process is enhanced. Evidence has been obtained that Mg^{2+} acts through lowering the affinity for ATP. In contrast to K^+, Mg^{2+} does not stimulate dephosphorylation in the absence of nucleotides. Mg^{2+} and nucleotides show the same interaction in the dephosphorylation process of a phosphoenzyme generated from inorganic phosphate. These findings suggest the presence of a low-affinity nucleotide binding site on the phosphoenzyme, as is found in the $(Na^+ + K^+)$-ATPase phosphoenzyme. This low-affinity binding site may function as a feed-back mechanism in proton transport.

Introduction

Recently we reported on the dephosphorylation behaviour of the $(K^+ + H^+)$-ATPase phosphoenzyme [1]. Dephosphorylation of the ^{32}P-labeled phosphoenzyme, initiated by adding millimolar ATP in the presence of micromolar Mg^{2+}, could be stimulated by ADP, giving direct evidence for the existence of an ADP-sensitive phosphoenzyme. A discrepancy was observed between the kinetics of dephosphorylation in the presence of either CDTA or millimolar ATP. In the presence of CDTA dephosphorylation was faster than with ATP and no stimulating effect of ADP was observed.

In the present paper we show studies on dephosphorylation of the $(K^+ + H^+)$-ATPase phosphoenzyme in which we have tried to elucidate the discrepancy between dephosphorylation in the presence of ATP and EDTA (or CDTA).

Materials and Methods

Enzyme preparation

A $(K^+ + H^+)$-ATPase containing membrane fraction is isolated from porcine gastric mucosa as described before [1]. The isolated preparation is stored at $-20°C$ in 0.25 M sucrose. The specific activity of the enzyme preparation ranges from 90

Abbreviation: CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid.

to 130 $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$ (determined according to Lowry, using bovine serum albumin as a standard) and the phosphorylation capacity with ATP ranges from 700 to 1200 $pmol \cdot (mg\ protein)^{-1}$.

Phosphorylation experiments

Phosphorylation with ATP is carried out by adding 50 μl membrane suspension (10 μg protein) to 50 μl phosphorylation medium. The final mixture contains 50 mM imidazole-HCl (pH 7.0), 7–15 μM Mg^{2+} (including 5 μM added Mg^{2+}) and 5 μM [γ-^{32}P]ATP. When other conditions are used this is mentioned in the text. After 10 s at 20°C the reaction is stopped and further processed as described before [1].

Phosphorylation with $^{32}P_i$ is carried out by adding 50 μl membrane suspension (10 μg protein) to 50 μl phosphorylation medium. The final mixture contains 50 mM imidazole-HCl (pH 7.0), 50 μM Mg^{2+} and 50 μM $^{32}P_i$. After 10 seconds at 20°C either the reaction is stopped immediately or dephosphorylation is initiated, by addition of 11 μl 20 mM non-radioactive P_i. Stopping and further processing of the samples is performed as described above.

Phosphorylation blanks are prepared by adding the enzyme to the stopping reagent, after which the phosphorylation medium is added.

Dephosphorylation of the $(K^+ + H^+)$-ATPase phosphoenzyme

Dephosphorylation of the ^{32}P-labeled enzyme is initiated in three different ways:

(1) Dilution of the tracer by addition of 11 μl 10 mM non-radioactive ATP plus possible additions to 100 μl incubation mixture.

(2) Complexation of Mg^{2+} by addition of 11 μl 10 mM EDTA plus possible additions.

(3) Substrate and cofactor dilution as in method 1 or 2, in combination with a 20-fold increase of the reaction volume.

The third method is used to study dephosphorylation in the presence of micromolar ATP concentrations. The concentrations mentioned in the text are all final concentrations. Dephosphorylation blanks are prepared by mixing phosphorylation- and dephosphorylation medium, after which the enzyme is added. After 20 s reaction the resulting ^{32}P-incorporation is determined as described above.

Calculations

Concentrations of free Mg^{2+}, free ATP and MgATP are calculated using stability constants determined in 0.1 M KCl at 20°C, given by Sillén and Martell [2]. For the equilibrium constants of the first and second protonation step of ATP^{4-} the values are 6.5 and 4.05 and for complexation of ATP^{4-} and $ATPH^{3-}$ with Mg^{2+} values of 4.00 and 2.00 are used.

Results

The main purpose of this study is to investigate the dephosphorylation process of the $(K^+ + H^+)$-ATPase phosphoenzyme. However we have first determined the K_m for ATP in the phosphorylation reaction in order to establish the minimal ATP concentration that is saturating to give full phosphorylation of all available sites.

Fig. 1 shows in a Scatchard plot the phosphorylation level as a function of the ATP concentration. For various enzyme samples a value for the

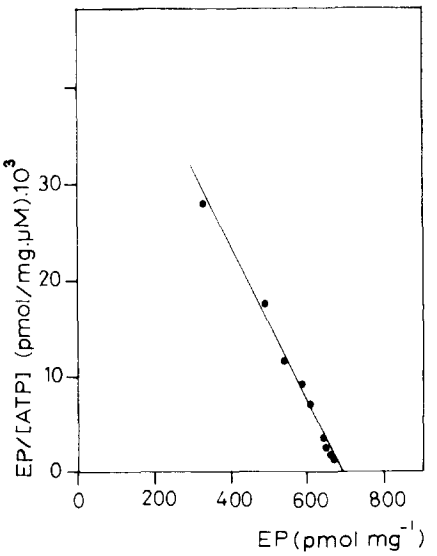


Fig. 1. ATP dependence of the phosphorylation reaction of $(K^+ + H^+)$-ATPase. $(K^+ + H^+)$-ATPase preparation (5 μg) is incubated for 10 s at 20°C in a 200 μl volume, containing 50 mM imidazole-HCl (pH 7.0), 1 mM Mg^{2+} and [γ-^{32}P]ATP in concentrations ranging from 0.02 to 1.0 μM. Further processing of the samples is as described in Materials and Methods.

K_m for ATP has been determined, giving an average value of 0.016 μM (S.E. = 0.002, $n = 5$). This value is at least 5-times lower than the K_m value of 0.1 μM reported by Ray and Forte [3].

Fig. 2 shows the dephosphorylation kinetics of the $(K^+ + H^+)$-ATPase phosphoenzyme in the presence of 1 mM EDTA, 1 mM ATP or 1 mM ATP plus 5 mM Mg^{2+}. The concentration of Mg^{2+} during phosphorylation is 7–15 μM. In the presence of ATP alone the dephosphorylation process is slow and monoexponential with a rate constant of 0.36 min^{-1}. From earlier studies [1,4] we know this occurs when the ADP-sensitive phosphoenzyme (E_1P) and the K^+-sensitive one (E_2P) dephosphorylate with the same rate constant, i.e., $k_3 = k_{-1}$ in Scheme I.

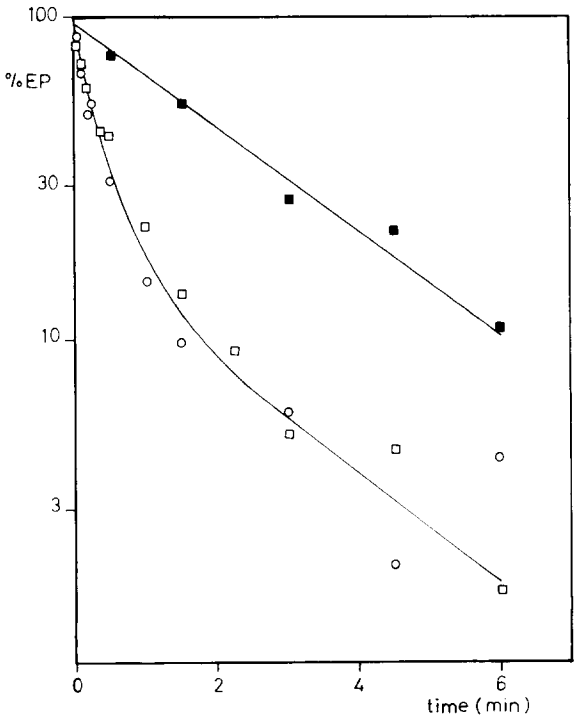


Fig. 2. Effects of EDTA, ATP and Mg^{2+} on the dephosphorylation rate. Steady-state phosphorylation levels are generated by treating the enzyme preparation for 10 s at 20°C in a medium containing 5 μM Mg^{2+}, 5 μM [γ-^{32}P]ATP and 50 mM imidazole-HCl (pH 7.0). Dephosphorylation is started by addition of 1 mM EDTA (○), 1 mM ATP (■) or 1 mM ATP plus 5 mM Mg^{2+} (□). After the desired length of time (10 s–6 min), dephosphorylation is terminated by the addition of the stopping reagent. Further processing of the samples is as described in Materials and Methods. 100% EP represents maximal phosphorylation capacity of the enzyme preparation.

$$\overset{k_{-1}}{\leftarrow} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \overset{k_3}{\rightarrow}$$

Scheme I.

In the presence of EDTA a curved line is obtained showing a fast phase (3.8 min^{-1}) and a slow phase (0.5 min^{-1}). Evidently in this condition k_3 and k_{-1} are not identical. The same curve is obtained in the presence of CDTA.

From Fig. 2 it becomes clear that dephosphorylation in the presence of ATP or EDTA are essentially different processes. It is important to establish whether EDTA stimulates the dephosphorylation process in an abnormal way or whether ATP in millimolar concentrations inhibits dephosphorylation. Therefore we studied the dephosphorylation process in the presence of 1 mM EDTA plus ATP in concentrations ranging from 5 μM to 5 mM (Fig. 3). Due to the presence of EDTA, phosphorylation is stopped sufficiently. The ATP concentration during phosphorylation is 0.5 μM. Fig. 3 shows that increasing the ATP concentration during dephosphorylation causes an increasing inhibition of the dephosphorylation process. A half-maximal concentration ($I_{0.5}$) for ATP of 0.1 mM is calculated. ADP has a similar inhibitory effect (data not shown) with an $I_{0.5}$ value of 0.7 mM.

Fig. 2 also shows the effect of Mg^{2+} on the dephosphorylation behaviour of the $(K^+ + H^+)$-ATPase phosphoenzyme. When 5 mM Mg^{2+} is present in addition to 1 mM ATP, the dephosphorylation process is the same as in the presence of EDTA. Increasing the Mg^{2+} concentration from 15 μM to 5 mM changes the kinetics of dephosphorylation gradually from the slow, monoexponential process, observed in the presence of ATP alone, to a fast double exponential process, like in the presence of EDTA. The stimulatory effect of Mg^{2+} is also observed when ADP is present instead of ATP.

In Figs. 2 and 3 two important phenomena are observed: (1) nucleotides inhibit the dephosphorylation process of the $(K^+ + H^+)$-ATPase phosphoenzyme, (2) Mg^{2+} abolishes the inhibitory effect of nucleotides.

We conclude that the inhibitory effect of

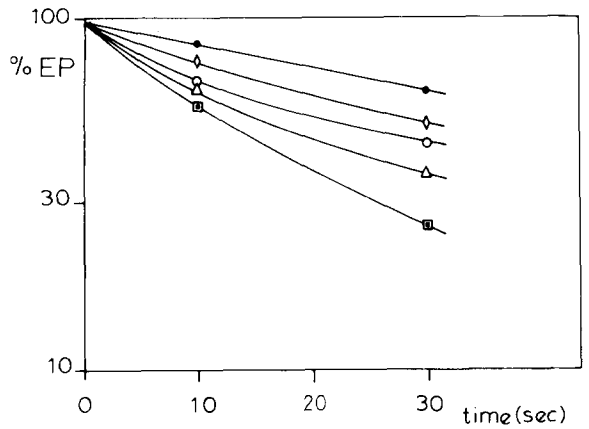


Fig. 3. Concentration dependence of the ATP-inhibition on dephosphorylation. Steady-state phosphorylation levels are generated by treating the enzyme preparation for 10 s at 20°C in a medium containing 5 μM Mg^{2+}, 0.5 μM [γ-^{32}P]ATP and 50 mM imidazole-HCl (pH 7.0). Dephosphorylation is started by addition of 1 mM EDTA (□) or 1 mM EDTA plus ATP in the concentrations: 0.005 mM (■), 0.05 mM (△), 0.1 mM (○), 0.2 mM (◇) or 5.0 mM (●). Dephosphorylation is terminated as described in the legend to Fig. 2. Rate constants for dephosphorylation are determined from phosphoenzyme levels after 10 s dephosphorylation. In this period of time dephosphorylation is linear under all circumstances of the experiment. $I_{0.5}$ values for ATP and ADP have been calculated from Scatchard plots showing the initial dephosphorylation rate as a function of the ATP or ADP concentration. 100% EP represents maximal phosphorylation capacity of the preparation.

nucleotides occurs through low-affinity binding ($[ATP]_{0.5} = 0.1$ mM) to a nucleotide binding site on the phosphoenzyme. Three possible explanations are proposed for the activating action of Mg^{2+} on dephosphorylation:

(1) Mg^{2+} complexes ATP and thus the concentration of free ATP is lowered. Free ATP would be an effective inhibitor and MgATP not or much less.

(2) Mg^{2+} decreases the ATP effect by lowering the affinity for ATP binding at the nucleotide site. According to this model an effect of Mg^{2+} on the $I_{0.5}$ value for ATP is expected.

(3) The effect of Mg^{2+} is not related to the presence of nucleotides. In other words Mg^{2+} exerts a general K^+-like effect by inducing a conformational change of the enzyme and thus promoting the hydrolysis of the phosphoenzyme.

To investigate the validity of explanation 1 we have measured the initial rate constants of dephosphorylation in the presence of three ATP concentrations (total concentrations) and varying Mg^{2+} concentrations (Fig. 4). These rate constants have been plotted against the concentrations of free Mg^{2+} (Fig. 4A), MgATP complex (Fig. 4B) and free ATP (Fig. 4C), which concentrations have been calculated using the stability constants for the MgATP complexes [2]. Possible binding of Mg^{2+} to the enzyme is not included in the calculations, but is not of much importance at the used enzyme concentrations (1–2 μM).

Fig. 4A shows the effect of free Mg^{2+} concentrations on the initial dephosphorylation rate. The half-effective concentration ($K_{0.5}$) of Mg^{2+} increases with increasing ATP concentration from 0.1 mM at 0.1 mM ATP to 0.24 mM at 1 mM ATP (Fig. 5A). Figs. 4B and 4C show that there is no simple relation between the dephosphorylation rate and the concentration of either MgATP or free ATP. At a specific concentration of free ATP, obtained by different combinations of total ATP and Mg^{2+}, different rate constants are obtained. Therefore the assumption that Mg^{2+} antagonizes the effect of ATP simply by complexing free ATP can not be valid.

Fig. 5B shows that the $I_{0.5}$ value for ATP increases with increasing Mg^{2+} concentration. In this experiment 10-s dephosphorylation levels have been taken, under which circumstances the rate of dephosphorylation is linear on a semilog scale. Moreover the $K_{0.5}$ value of Mg^{2+} increases with increasing ATP concentration. The antagonistic effects of Mg^{2+} and ATP are in line with explanation 2. However since K^+ has similar antagonistic effects towards ATP (Figs. 5A and 5B), explanation 3 is not excluded.

K^+ not only affects binding of nucleotides to the enzyme but also induces a conformational change which leads to stimulation of dephosphorylation and is also observed in the absence of ATP [5,6]. We therefore would like to study the effect of Mg^{2+} on dephosphorylation in the absence of nucleotides.

Two methods exist to initiate dephosphorylation: (1) Dilution of [γ-^{32}P]ATP by addition of non-radioactive ATP, after which dephosphorylation of the ^{32}P-labeled enzyme can be studied. (2) Chelation of Mg^{2+} ions, which are essential for phosphorylation. In this way phosphorylation

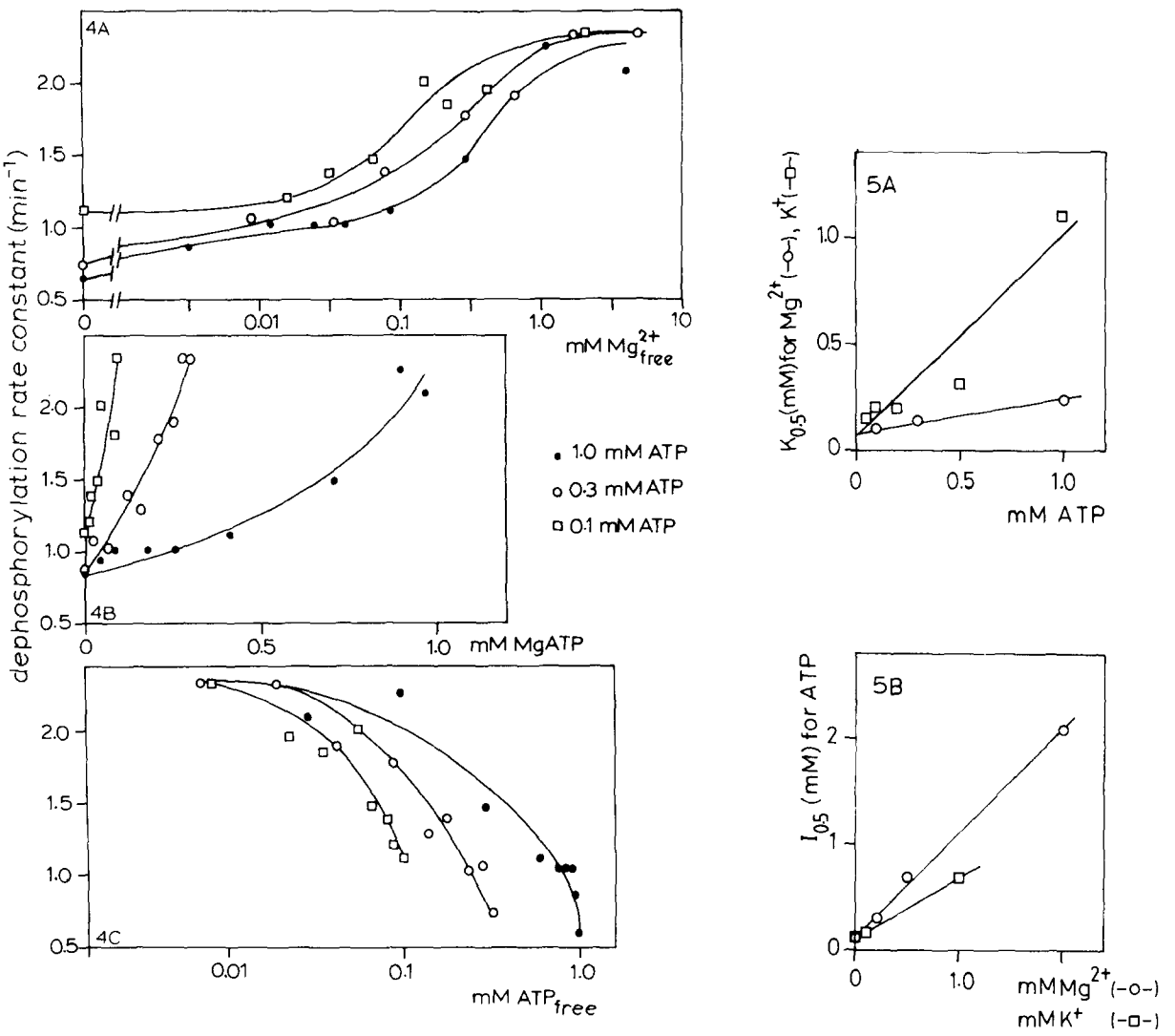


Fig. 4. Dephosphorylation of the $(K^+ + H^+)$-ATPase phosphoenzyme in the presence of various ATP and Mg^{2+} concentrations. Steady-state phosphorylation levels are generated as described in the legend to Fig. 2. Dephosphorylation is initiated by addition of 0.1 mM ATP (□), 0.3 mM ATP (○) or 1.0 mM ATP (●) (final concentrations of total ATP present). Mg^{2+} is included in the dephosphorylation mixture in concentrations, ranging from 0 to 5 mM. Rate constants for dephosphorylation are calculated as described in the legend to Fig. 3. Dephosphorylation rate constants are plotted against concentrations of free Mg^{2+} (A), MgATP (B) and of free ATP (C), which values have been calculated by use of stability constants for the MgATP complexes as described in Materials and Methods.

Fig. 5. Mutual effects of K^+ and Mg^{2+} vs. ATP on the dephosphorylation process. Phosphoenzyme is generated from ATP as described in the legend to Fig. 2. Dephosphorylation is initiated by addition of ATP in concentrations from 0.05 to 1.0 mM, in the presence of various Mg^{2+} (○) or K^+ concentrations (□). $K_{0.5}$ values have been calculated from Scatchard plots showing the initial dephosphorylation rates as a function of the Mg^{2+}, K^+ or ATP concentrations. Initial rate constants for dephosphorylation in the presence of Mg^{2+} have been calculated as described in the legend to Fig. 3. For the K^+ stimulated dephosphorylation initial rate constants have been calculated by taking 2-s dephosphorylation levels. In this period dephosphorylation is not quite linear on a semilog plot, especially not at high K^+-concentrations. The actual $K_{0.5}$ values will thus differ slightly from the calculated values. (A) $K_{0.5}$ values for Mg^{2+} and K^+ plotted as a function of the ATP concentration. (B) $I_{0.5}$ values for ATP plotted as a function of the Mg^{2+} or K^+ concentration.

stops completely. Considering the nature of the two methods it is obviously impossible to study the effect of Mg^{2+} in the absolute absence of nucleotides. Therefore we have initiated dephosphorylation by adding a micromolar concentration of ATP (method 3, Materials and Methods).

Fig. 6 shows the dephosphorylation of a phosphoenzyme, generated from 0.25 μM ATP, a concentration which is low, but saturating to give the maximal steady-state phosphorylation level (Fig. 1). Dephosphorylation is initiated by 20-fold dilution of the 50 μl phosphorylation medium in combination with tracer dilution or Mg^{2+} complexation (method 3 in Materials and Methods). The concentration of non-radioactive ATP used during dephosphorylation is 5 μM. As a consequence [γ-^{32}P]ATP is diluted sufficiently (20 × 20 = 400 times) during dephosphorylation to enable studying this process correctly. Fig. 6 clearly shows that dephosphorylation in the presence of 2 mM

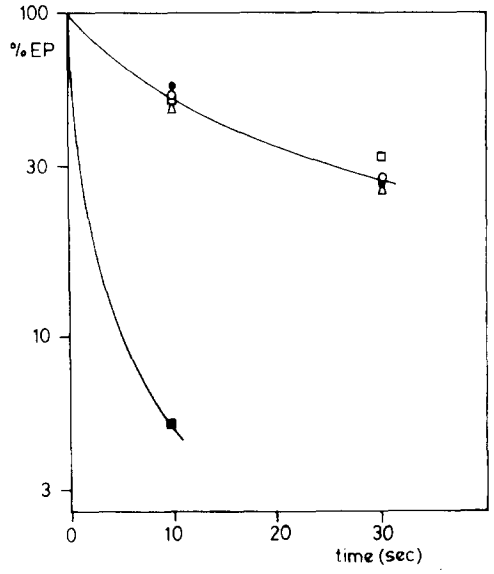


Fig. 6. Effect of various ligands on dephosphorylation in the presence of micromolar ATP. Phosphoenzyme is generated by incubating ($K^+ + H^+$)-ATPase (5 μg) in a 50 μl volume containing 50 mM imidazole-HCl (pH 7.0), 1 μM Mg^{2+}, 0.25 μM [γ-^{32}P]ATP. Dephosphorylation is initiated by addition of a volume of 950 μl, containing 2 mM EDTA (○), 5 μM ATP (●), 5 μM ATP plus 0.1 mM EDTA (□), 5 μM ATP plus 10 mM Mg^{2+} (△), 5 μM ATP plus 5 mM K^+ (■). Stopping of the reaction and further processing of samples is as described in the legend to Fig. 2. 100% EP represents maximal phosphorylation capacity of the enzyme preparation.

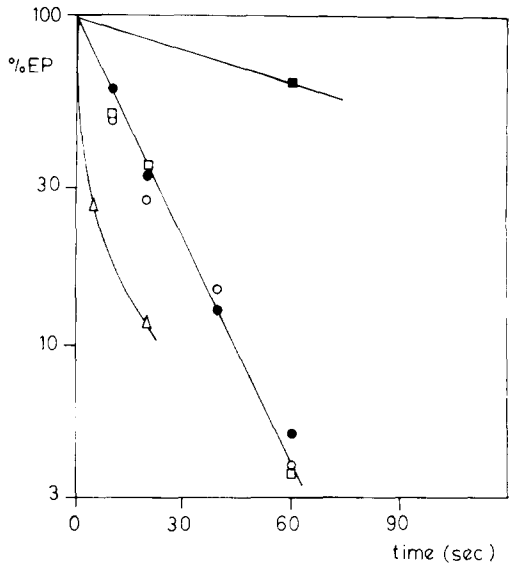


Fig. 7. Effect of various ligands on dephosphorylation of a phosphoenzyme, generated from P_i. Phosphoenzyme is generated by incubating a ($K^+ + H^+$)-ATPase preparation (10 μg) in a volume of 100 μl, containing 50 mM imidazole-HCl (pH 7.0), 50 μM Mg^{2+} and 50 μM P_i. After 10 s at 20°C dephosphorylation is initiated by addition of 2 mM P_i (●), 2 mM P_i + 5 mM EDTA (○), 2 mM P_i + 5 mM Mg^{2+} (□), 2 mM P_i + 5 mM ATP (■) or 2 mM P_i + 5 mM K^+ (△). Stopping of the reaction and further processing of the samples is as described in the legend to Fig. 2. 100% EP represents a ^{32}P-incorporation of 420 pmol per mg protein.

EDTA, 5 μM ATP alone or 5 μM ATP plus 0.1 mM EDTA is the same, suggesting that ^{32}P-incorporation is stopped sufficiently and that a concentration of 5 μM ATP is not inhibiting. The latter observation is not surprising as we determined an $I_{0.5}$ value of 0.1 mM for the inhibitory effect of ATP. When 10 mM Mg^{2+} is included with 5 μM ATP, no significant stimulation is observed, while K^+ has an accelerating effect (Fig. 6). Hence it is concluded that the stimulatory effect of Mg^{2+} in contrast to that of K^+, depends on the presence of nucleotides and the effect of Mg^{2+} cannot be a general K^+-like effect as explanation 3 assumes.

More evidence for this conclusion is obtained in the experiment of Fig. 7, which shows the dephosphorylation kinetics of a ($K^+ + H^+$)-ATPase phosphoenzyme, generated from 50 μM P_i in the presence of 50 μM Mg^{2+}. It is shown that ATP also stabilizes this phosphoenzyme ($I_{0.5}$ =

0.45 mM). Mg^{2+} here also abolishes the ATP effect (not shown). When dephosphorylation is initiated with 2 mM P_i no difference in dephosphorylation behaviour is observed whether 50 μM Mg^{2+}, 5 mM EDTA or 5 mM Mg^{2+} are present. On the other hand K^+ stimulates the dephosphorylation. Thus in the absence of nucleotides Mg^{2+} does not, in contrast to K^+, stimulate dephosphorylation of a phosphoenzyme, generated from either ATP or P_i.

Discussion

This paper shows two important phenomena, which may help to elucidate the mechanism of action of the $(K^+ + H^+)$-ATPase. Firstly, evidence is given for low-affinity binding of nucleotides to the phosphoenzyme and secondly Mg^{2+} is able to abolish this effect, thus stimulating dephosphorylation.

The reduction of the dephosphorylation rate of the $(K^+ + H^+)$-ATPase phosphoenzyme by ATP demonstrates that ATP binds to the phosphoenzyme. That the ATP effect is functionally significant and not due to non-specific binding is indicated by the different efficacies of the tested nucleotides. $I_{0.5}$ values of 0.1 mM ATP and 0.7 mM ADP have been determined.

In studies on $(Na^+ + K^+)$-ATPase similar phenomena have been observed. Askari and Huang [7] have reported inhibition of dephosphorylation of a $(Na^+ + K^+)$-ATPase phosphoenzyme, generated from P_i, by nucleotides on a low-affinity site. The phosphoenzyme generated from P_i in the studies of Askari and Huang is neither K^+ nor ADP sensitive, but as phosphorylation by P_i is at the same site as with ATP, the reported phenomena may nevertheless be similar to our findings. Moreover Fukushima et al. [8] have observed stabilization of a phosphoenzyme, generated from ATP, by nucleotides on a low-affinity site. In their studies Mg^{2+} also decreased the nucleotide effect, as observed for the $(K^+ + H^+)$-ATPase. In both papers the reported phenomena have been correlated with the low-affinity for ATP in the overall K^+-stimulated reaction, although the results could not completely be explained in this fashion. With respect to $(K^+ + H^+)$-ATPase it seems logical to make the same correlation.

The $I_{0.5}$ for ATP in the dephosphorylation of the $(K^+ + H^+)$-ATPase phosphoenzyme is similar to the K_m of the low-affinity ATP effect, detected by kinetic studies on the K^+-stimulated Mg^{2+}-ATPase activity of $(K^+ + H^+)$-ATPase (0.2 mM, Ref. 9). A low affinity for ATP and ADP has also been observed in studies on the ATP/ADP exchange reaction (0.12 mM, Ref. 10). However it seems paradoxical that ATP binding to the phosphoenzyme stabilizes it, while the overall ATPase reaction is enhanced at a low-affinity site.

In this regard it is important to recall that in previous studies, inhibition by high ATP on the overall ATPase reaction has been reported [11]. From the published data we roughly estimate a $K_{0.5}$ value of 0.1 mM for the inhibition. This apparently high value has been determined in the presence of 20 mM K^+ and 3 mM Mg^{2+}, both of which have been shown to decrease the affinity for ATP (Fig. 5). High ATP concentrations inhibit also the ATP/ADP exchange reaction, probably in the same manner. $K_{0.5}$ values for the inhibition are estimated by us to 0.5 mM [10] and 0.3 mM [12].

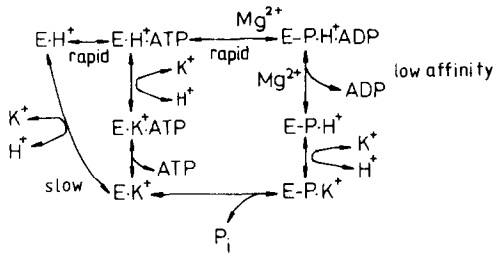
The second important observation in this paper is the fact that Mg^{2+} counteracts the inhibitory effect of nucleotides. It appears unlikely that Mg^{2+} acts through complexing ATP: no direct relationship between free ATP and the initial dephosphorylation rate exists. The $I_{0.5}$ value for ATP decreases with increasing Mg^{2+} (and K^+). This is not merely an effect of ionic strength as we did not observe any effect with choline. We assume that the low-affinity binding of nucleotides is hindered by the binding of Mg^{2+}. In this way Mg^{2+} and K^+ act similarly. However Mg^{2+} does not act similar to K^+ in all aspects: only K^+ directly stimulates dephosphorylation in the absence of nucleotides. Mg^{2+} is not able to perform the same action.

Antagonistic effects of K^+ and ATP and of Mg^{2+} and ATP have been reported before. Schrijen et al. [13] observed in studies on butanedione-inactivation that K^+ and Mg^{2+} lower the protective action of nucleotides against inactivation. In a study on inactivation of the $(K^+ + H^+)$-ATPase by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) it has been observed that ATP protects against the K^+-stimulated enhancement of in-

activation [14]. Moreover K^+ lowers the K_m for ATP in the net transport process [15] and the overall ATPase reaction [16].

The findings in this paper shed new light on the fact that an ADP-sensitive phosphoenzyme could not be detected initially. ADP can have two effects on dephosphorylation: (1) a stimulatory effect which is detected when the rate constants of hydrolysis of the two phosphoenzymes (k_3 and k_{-1}) are equal [1]; (2) an inhibitory effect as shown in this paper, which is a general nucleotide effect. This effect can be observed when dephosphorylation is initiated in the presence of either CDTA or millimolar ATP plus Mg^{2+}. It might be that under those conditions, generally used by other authors [5,6,17] the stimulatory effect of ADP is overruled by the inhibitory effect of ADP.

From the presented results on interaction of Mg^{2+}, ATP and K^+ with the $(K^+ + H^+)$-ATPase the following reaction sequence is summarized (adapted from the model proposed by Ljungström et al. [9,15]. Much of the evidence for this model (Scheme II) has been published elsewhere [6,9,16,17].



Scheme II.

The first step in the sequence is binding at the enzyme of a cytosolic proton, forming a complex which binds ATP at a high affinity site. After binding of Mg^{2+}, ATP breaks down forming an $E\text{-}P \cdot H^+ \cdot ADP$ comformation of the enzyme. Whether Mg^{2+} remains bound at the enzyme has not been proven as yet. The enzyme is phosphorylated at an aspartyl residue [18]. Evidence for this step and the subsequent release of ADP is the occurrence of an ATP/ADP exchange reaction [10,12]. An ADP sensitive phosphoenzyme (E_1P) has recently been detected [1].

ADP leaves the phosphoenzyme from a low-affinity site while H^+ remains bound [17]. As proposed by Scarborough [19] E_1P is the conformation of the phosphoenzyme from which ADP has dissociated but which has not yet moved into such a conformation to make hydrolysis of the aspartyl phosphate possible. We assume stabilization of the phosphoenzyme by nucleotides occurs in this stage of the reaction cycle. Nucleotides bind at the low-affinity nucleotide site, thus hindering the next step. Mg^{2+} and K^+ decrease the affinity for nucleotides and consequently stimulate dephosphorylation.

The overall ATPase reaction measured as the release of P_i, is a complex reaction as substrate (ATP) and cofactors involved (K^+, Mg^{2+} and H^+) have both stimulating and inhibiting effects. Optimal conditions for the ATPase reaction are obtained at such a pH and K^+-concentration, which provide sufficient turnover of the phosphoenzyme, while the rate of phosphorylation is not too far decreased [9]. It is assumed that at the optimal K^+ concentration the rate of phosphorylation is rate limiting for the overall ATPase reaction [6]. Mg^{2+} plays its own specific role in this process. It is essential for phosphorylation [3] and stimulates dephosphorylation as shown in this paper. At high ATP/Mg^{2+} ratios, ATP will bind to a low-affinity site on the phosphoenzyme and will thus slow down the release of P_i. Thus it functions as a control mechanism at the $E\text{-}P \cdot H^+$ rather than the $E \cdot K^+$ stage. Actually inhibition at the $E\text{-}P \cdot H^+$ level may serve as a feed-back mechanism in proton transport.

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