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POTASSIUM-*p*-NITROPHENYL PHOSPHATE INTERACTIONS WITH ($Na^+ + K^+$)-ATPase

THEIR RELEVANCE TO PHOSPHATASE ACTIVITY

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The K^+-dependent *p*-nitrophenylphosphatase activity catalyzed by purified ($Na^+ + K^+$)-ATPase from pig kidney shows substrate inhibition (K_i about 9.5 mM at 2.1 mM Mg^{2+}). Potassium antagonizes and sodium favours this inhibition. In additon, K^+ reduces the apparent affinity for substrate activation, whereas *p*-nitrophenyl phosphate reduces the apparent affinity for K^+ activation. In the absence of Mg^{2+}, *p*-nitrophenyl phosphate, as well as ATP, accelerates the release of Rb^+ from the Rb^+ occluded unphosphorylated enzyme. With no Mg^{2+} and with 0.5 mM KCl, trypsin inactivation of ($Na^+ + K^+$)-ATPase as a function of time follows a single exponential but is transformed into a double exponential when 1 mM ATP or 5 mM *p*-nitrophenyl phosphate are also present. In the presence of 3 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate and without KCl the trypsin inactivation pattern is that described for the E_1 enzyme form; the addition of 10 mM KCl changes the pattern which, after about 6 min delay, follows a single exponential. These results suggest that (i) the shifting of the enzyme toward the E_1 state is the basis for substrate inhibition of the *p*-nitrophenylphosphatase activity of($Na^+ + K^+$)-ATPase, and (ii) the substrate site during phosphatase activity is distinct from the low-affinity ATP site.

Introduction

The K^+-dependent ouabain-and-vanadate-sensitive phosphatase activity is a biochemical reaction catalyzed by ($Na^+ + K^+$)-ATPase, even by those enzyme preparations obtained in a very high purity, which does not seem to be associated with cation translocation [1–5]. In this reaction the information about the actual mechanism of hydrolysis is fragmentary, and although it is believed that there is a relation with the ($Na^+ + K^+$)-ATPase activity, the nature of that relationship is not completely understood. Among the different compounds that can act as phosphatase substrate, *p*-nitrophenyl phosphate is the most commonly used [1–5]. Besides K^+, Mg^{2+} appears as an essential activator whereas Na^+ acts as inhibitor reducing the apparent affinity for K^+ [6–9]. In addition, there are some interesting, and intriguing, interactions taking place between the enzyme, *p*-nitrophenyl phosphate and K^+. An increase in K^+ concentration leads to an increase in the maximal rate of hydrolysis but also to a reduction in the apparent affinity for the substrate [6,8,10]; on the other hand, the reaction shows substrate inhibition, an effect that is antagonized by K^+ [6–8]. The aim of the present work was precisely to look, in more detail, into the possible mechanism of substrate inhibition of the *p*-nitrophenylphosphatase activity.

Methods

The experiments were performed on ($Na^+ + K^+$)-ATPase partially purified from pig kidneys according to the method of Jørgensen [11]. The

specific activity initially obtained was between 12 and 15 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ and decayed at a rate of about 10% per month when the enzyme was stored at 0 °C. Phosphatase activity was measured at 37 °C using *p*-nitrophenyl phosphate as a substrate; the reaction was stopped with 1 vol. of 0.2 M NaOH/2.5% SDS/4 mM EDTA and the production of *p*-nitrophenol was estimated by the absorbance at 410 nm [12]. ATPase activity was assayed determining the release of $^{32}P_i$ from [γ-^{32}P]ATP by extraction with isobutanol/benzene; the labelling of the [γ-^{32}P]ATP was performed according to Glynn and Chappell [13] as modified by De Meis [14]. In these experiments protein was determined by the method of Lowry et al. [15] with the modification of Markwell et al. [16].

The occlusion of Rb^+ in ($Na^+ + K^+$)-ATPase was estimated under equilibrium and transient conditions. (a) Equilibrium experiments: the procedure was essentially similar to that described by Beaugé and Glynn [17] and is detailed in the legend to Fig. 3. The enzyme suspension was passed through a Dowex 50 W X-8 (50–100 mesh) resin column and the effluent collected in a scintillation vial containing 3 ml distilled water; the vial was counted in a Beckman scintillation counter with automatic quenching correction using the Tritium channel. The vial was then cooled in an ice bath adding 0.3 ml of 55% trichloroacetic acid and allowed to stand for 10–15 min. The denatured protein solution was passed through a Whatman GF/C filter and the protein trapped in the filter was determined after staining with Ponceau-S; the procedure was as described in Ref. 18 except that the solution with the disrupted filter was centrifuged at 12000 × *g* for 2 min in an Eppendorf centrifuge prior to reading the absorbance at 520 nm. Bovine serum albumin was used as a standard. (b) Transient experiments: these were performed as described in Ref. 19 utilizing a small volume (about 0.1 ml) of Sephadex G-25 on top of the Dowex resin as a rapid mixing chamber. The experimental details are given in the legend to Table I.

Trypsin inactivation was performed as described by Jørgensen [20]; digestion was stopped by the addition of soybean trypsin inhibitor in a 4 : 1 ratio of inhibitor to trypsin.

All solutions were made with bidistilled deionized water. The NaCl and KCl salts were Baker Ultrex; all other chemicals were reagent grade. ADP, ATP (sodium salts), *p*-nitrophenyl phosphate (Tris salt), trypsin and soybean trypsin inhibitor were obtained from Sigma, U.S.A. The nucleotides were transformed into Tris salts after being passed through Dowex 50 columns. Rubidium-86 was purchased from the Comisión Nacional de Energía Atómica of Argentina as RbCl. The solution composition varied in the different experiments and is given in the corresponding figure and table legends. Unless otherwise stated all experimental points correspond to the means of triplicate determinations and each experiment was repeated at least once.

Mg^{2+} concentrations were calculated taking a value of 10 mM for the Mg-*p*-nitrophenyl phosphate dissociation constant at 37 °C and 150 mM ionic strength.

Results

Experiments of K^+-dependent p-nitrophenyl-phosphatase activity

Preliminary experiments (not shown) confirmed earlier reports on other ($Na^+ + K^+$)-ATPase preparations about substrate inhibition of *p*-nitrophenyl phosphatase activity as well as the protection of K^+ against this effect [6–8]. In our case inhibition developed, with the resiprocal plots bending upward at low 1/[*p*-nitrophenyl phosphate], at 1 mM KCl but fail to appear when the concentration of KCl was 20 mM.

Fig. 1 is an experiment performed to follow *p*-nitrophenyl phosphatase activity as a function of substrate concentration over a non-inhibitory range at different concentrations of KCl. The activation curve obeys Michaelian kinetics under all conditions investigated; in addition, an increase in KCl concentration results in a higher maximal velocity as well as in a lower apparent affinity for the substrate: the K_m for *p*-nitrophenyl phosphate went from 1.2 mM to 5 mM when the concentration of KCl was increased from 1 mM to 5 mM. In addition, a replot of the data from Fig. 1 (not shown) indicates that (i) a sigmoid relationship exists between KCl concentration and *p*-nitrophenylphosphatase activity, and (ii) an increase in the concentration of *p*-nitrophenyl phos-

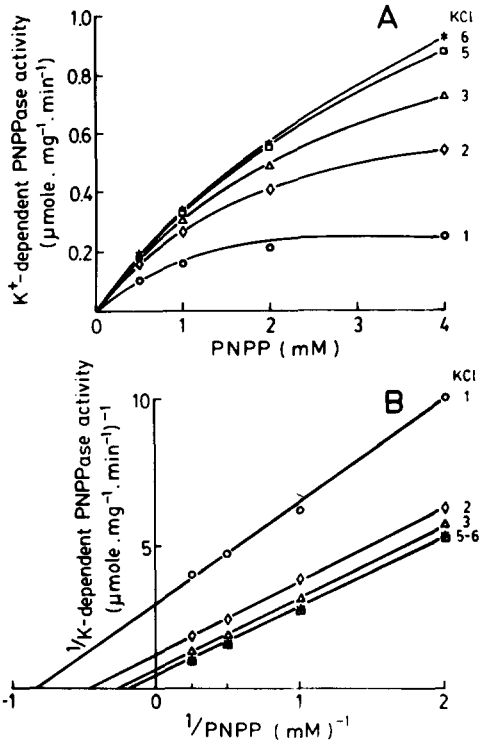


Fig. 1. (A) Effects of different KCl concentrations on the substrate activation curve of the K^+-dependent *p*-nitrophenylphosphatase activity of purified ($Na^+ + K^+$)-ATPase. The composition of the incubation solutions was the following (mM): Tris-HCl (pH at 37 °C, 7.4), 180 − KCl concentration; EGTA, 0.1: Mg^{2+}, 2.1; *p*-nitrophenyl phosphate (PNPP) 0.5 to 4.0; KCl, 1 (○——○), 2 (◇——◇), 3 (△——△), 5 (□——□) or 6 (*——*). (B) Double reciprocal plots of the data from (A). All points correspond to means of duplicate determinations. Note that an increase in KCl leads to an increase in the maximal velocity and a reduction in the apparent affinity for the substrate. See text for details.

phate leads to a reduction in the apparent affinity for KCl. The $K_{0.5}$ for KCl went from 1 mM to 1.7 mM when *p*-nitrophenyl phosphate concentration was increased from 0.5 mM to 4 mM.

The effect of high concentrations of *p*-nitrophenyl phosphate (from 10 to 50 mM) on the phosphatase activity at different KCl concentrations is illustrated in Fig. 2. Above 20 mM *p*-nitrophenyl phosphate substrate inhibition is observed at all K^+ concentrations investigated (1 to 5 mM), but the magnitude of that inhibition is decreased as K^+ concentration increases. In addition, the simultaneous presence of NaCl has an effect similar to that of lowering the concentration of K^+. The Dixon plots of Fig. 2B, which include data only in the range of substrate concentrations that are inhibitory, are all linear and give a K_i for *p*-nitrophenyl phosphate of 9.5 mM.

Experiments on rubidium occlusion

We have seen that substrate inhibition of *p*-nitrophenyl phosphatase activity is antagonized by K^+ and favoured by Na^+. On the other hand, the current idea about phosphatase activity of (Na^+ +

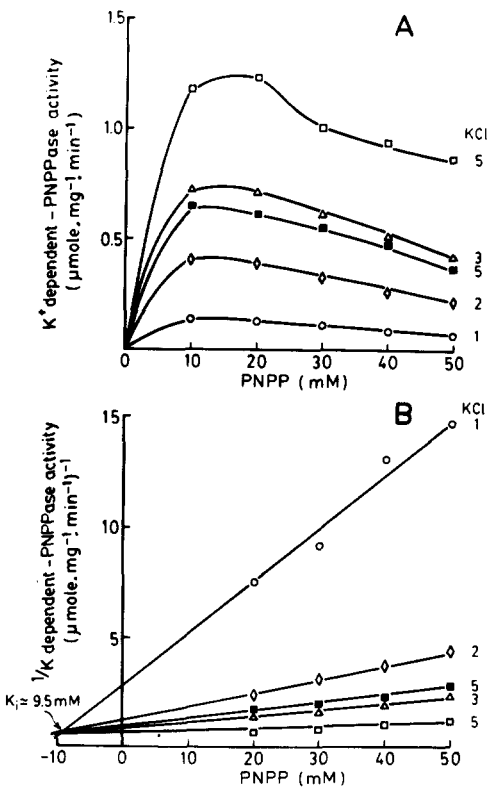


Fig. 2. Effects of different KCl concentrations, either alone (open symbols) or with NaCl (filled symbols) on substrate inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity of purified ($Na^+ + K^+$)-ATPase. The composition of incubation solutions was the following (mM): Tris-HCl (pH at 37 °C, 7.4), 180 − (KCl + NaCl) concentration; EGTA, 0.1; Mg^{2+}, 2.1; *p*-nitrophenyl phosphate (PNPP), 10 to 50; KCl, 1 (○——○), 2 (◇——◇), 3 (△——△) or 5 (□——□). Filled squares correspond to 5 mM KCl plus 10 mM NaCl. All points are the means of duplicate determinations. (A) Direct plots. (B) Dixon plots. Note: (i) all Dixon plots are linear; (ii) KCl protects whereas NaCl favours substrate inhibition; (iii) the K_i for *p*-nitrophenyl phosphate is about 9.5 mM. See text for details.

K^+)-ATPase is that it involves the enzyme in the $E_2(K)$ form [10,23–26]. If this idea is correct, one way to account for substrate inhibition is that *p*-nitrophenyl phosphate can displace the enzyme away from the $E_2(K)$ occluded state. This possibility was initially explored in equilibrium experiments where the enzyme was first allowed to occlude Rb^+ and then exposed to different concentrations of ATP or *p*-nitrophenyl phosphate. The amount of Rb bound to the enzyme after passing through the cation exchange column was compared with that remaining when 2 mM ADP were present in the incubation solution. Assuming that 2 mM ADP induce full disocclusion [17,19] Fig. 3 can be taken as a dose-response curve of the disoccluding ability of the tested ligands. From the figure, the $K_{0.5}$ is about 3 μM for ATP (A) and around 250 μM for *p*-nitrophenyl phosphate (B).

Apart from accelerating the disocclusion rate, there are two other ways *p*-nitrophenyl phosphate could shift the $E_2(K)$-E_1K equilibrium in favour of E_1K: (i) chelating free Rb, and (ii) inhibiting the rate of $E_2(K)$ formation. To rule out these possibilities $E_2(Rb)$ occluded enzyme suspension was exposed to the tested ligands immediately before entering the cation exchange column. This was achieved as described in Ref. 19 including 0.1 ml of Sephadex equilibrated with the desired ligand on top of the Dowex column; the function of the Sephadex was to act as a rapid mixing chamber.

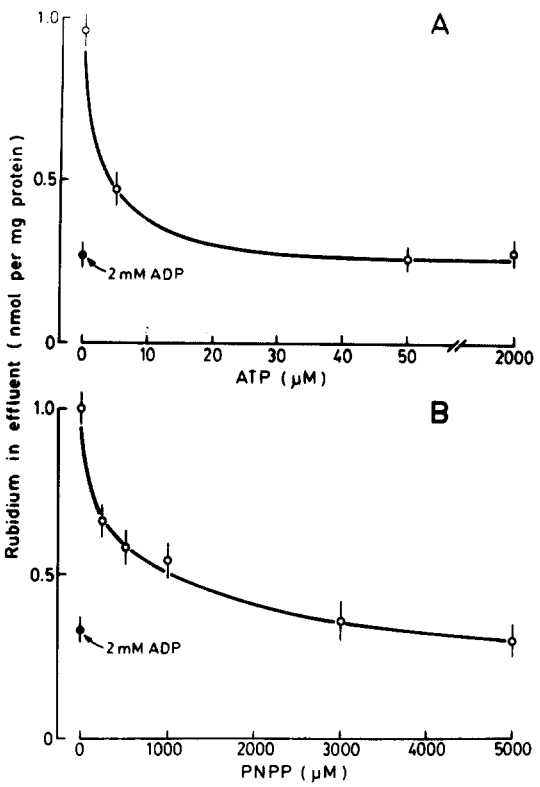


Fig. 3. Release of occluded rubidium from unphosphorylated purified ($Na^+ + K^+$)-ATPase at equilibrium with different concentrations of ATP (A) or *p*-nitrophenyl phosphate (PNPP) (B). Enzyme aliquots (0.25 mg with an specific activity of 10.4 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$) was incubated in 0.5 ml of a solution with the following composition (mM): Tris-HCl, (pH at 20 °C, 7.4), 100; EDTA-Tris, 0.5; ^{86}RbCl, 0.1. After 1 min different concentrations of ATP (A) or *p*-nitrophenyl phosphate (B) were added, and after another 30 s the mixture was passed through a Dowex 50 X-8 (100) column (0.5 ml total volume) preequilibrated with the buffer and EDTA; the average time the enzyme was in contact with the column was 0.7. Radioactivity and total protein content in the effluent were estimated as described in Methods. All points correspond to the means ±S.E. of triplicate determinations. The amount of occluded rubidium was taken as the difference between the rubidium retained in the effluent in the absence and presence of 2 mM ADP in the incubation solution.

TABLE I

RELEASE OF Rb^+ FROM RUBIDIUM-OCCLUDING UNPHOSPHORYLATED ($Na^+ + K^+$)-ATPase DURING A TRANSIENT EXPOSURE TO ATP OR *p*-NITROPHENYL PHOSPHATE (PNPP)

The enzyme suspension consited of Tris/Tris-HCl (pH 7.4 at 20 °C), 100 mM; EDTA-Tris, 0.5 mM; ^{86}RbCl, 0.1 mM; enzyme (spec. act. 10.4 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$) 0.25 mg, in a total volume of 0.5 ml. The suspension was equilibrated for 1 min before it was passed through the resin. The ligand concentration in the Sephadex is the concentration in the solution with which the Sephadex was equilibrated; the concentration attained in the effluent mixtures, assuming uniform mixing, corresponds to about 4% of that in the Sephadex. Each value is the mean ±S.E. of triplicate determinations. For details see Methods, legend to Fig. 3 and Ref. 19.

Ligand in enzyme suspension (mM)	Ligand in Sephadex (mM)	Rubidium remaining in effluent after 0.7s	
		Total minus ADP ($nmol \cdot mg^{-1}$)	%
–	–	0.60 ± 0.05	100
2 ADP	2 ADP	0.0	0
–	50 ATP	−0.02 ± 0.03	0
–	50 PNPP	0.48 ± 0.04	80
–	200 PNPP	0.30 ± 0.05	50

Because during the passage through the Dowex resin practically all free Rb^+ is trapped by the exchanger, the amount of Rb^+ released from the enzyme during that time can be taken as an expression of the actual disocclusion rate. The results of these experiments, summarized in Table I, indicate that *p*-nitrophenyl phosphate indeed accelerates the rate of disocclusion of the enzyme-Rb complex (although some inhibition of E_2(Rb) formation cannot be completely disregarded). In addition, considering that the average concentration of the ligands in the enzyme suspension is about 4% of that in the Sephadex, Table I also shows that *p*-nitrophenyl phosphate exerts its effect acting with a rather low apparent affinity ($K_{0.5}$ in the mM range). We will come back to this point in the Discussion.

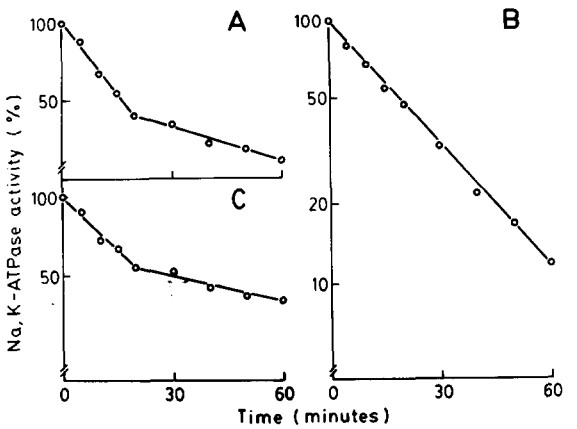
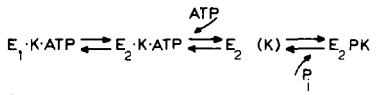


Fig. 4. Effects of potassium and of potassium plus *p*-nitrophenyl phosphate on trypsin inactivation of purified $(Na^+ + K^+)$-ATPase in the absence of magnesium. Aliquots of the enzyme containing 0.1 mg protein were incubated at 37 °C in 1 ml with 25 mM imidazole (pH at 37 °C, 7.5), 150 mM choline chloride with no other ligand (A), in the presence of 0.5 mM KCl (B) or with 0.5 mM KCl plus 5 mM *p*-nitrophenyl phosphate (C). Digestion was started by the addition of trypsin (final concentration 3 μg/ml) and was stopped by mixing 50 μl of the incubation mixture with 20 μl of 25 mM imidazole (pH 7.5) containing 12 μg of trypsin inhibitor. After completion of incubation $(Na^+ + K^+)$-ATPase activity was assayed in a medium containing (mM): $MgCl_2$, 3; ATP, 3; imidazole (pH at 37 °C, 7.4), 25; KCl, 20; NaCl, 130 and EGTA, 0.1. Note: (i) in the absence of KCl inactivation follows a pattern described for enzyme in the E_1 form; (ii) the addition of 0.5 mM KCl leads to a diggestion pattern of the E_2 state, and (iii) the pattern becomes E_1 when 5 mM *p*-nitrophenyl phosphate are included with 0.5 mM KCl. For details see text and Ref. 20.

Experiments on trypsin digestion

The generally accepted scheme for $(Na^+ + K^+)$-dependent ATP hydrolysis [1–5] implies that there are two pathways by which the enzyme-K complex can be removed from its occluded state:



Scheme I.

(i) going into the unphosphorylated $E_1 \cdot K \cdot ATP$ complex (ATP effect), and (ii) going into the phosphorylated E_2PK form (P_i effect). The fact that P_i inhibits phosphatase activity by competing with *p*-nitrophenyl phosphate [1–5], whereas it does not bind to the enzyme in the absence of Mg^{2+} [21] might suggest that *p*-nitrophenyl phosphate acts like ATP; nevertheless a P_i-like effect cannot be a priori ruled out. To clarify this point trypsin inactivation of the $(Na^+ + K^+)$-dependent ATP hydrolyses as a function of time was followed in enzyme incubated in 150 mM choline chloride-buffered solutions containing no potassium, KCl alone and KCl plus *p*-nitrophenyl phosphate. The results of one of these experiments are illustrated in Fig. 4. The inactivation pattern typical of enzyme in the E_1 form found in K^+-free choline (A) (20) was transformed in that corresponding to the E_2 state (20) by 0.5 mM KCl (B), but was returned to the E_1 type when in addition to 0.5 mM KCl there were also 5 mM *p*-nitrophenyl phosphate in the digestion solution (C). Similar results were found when 1 mM ATP was used instead of the phosphatase substrate (not shown). This is another indication of the disoccluding ability of *p*nitrophenyl phosphate with the additional information that that disocclusion takes place by pushing the enzyme into the E_1 comformation in an ATP-like effect.

In the presence of 150 mM choline chloride and 3 mM $MgCl_2$ trypsin inactivation of $(Na^+ + K^+)$-dependent ATP hydrolysis corresponds to E_1 pattern [27]; under these conditions addition of KCl brings it into that corresponding to the E_2 state (Beaugé and Pedemonte, unpublished results). It is of obvious importance to find what happens when both Mg^{2+} and *p*-nitrophenyl phosphate are

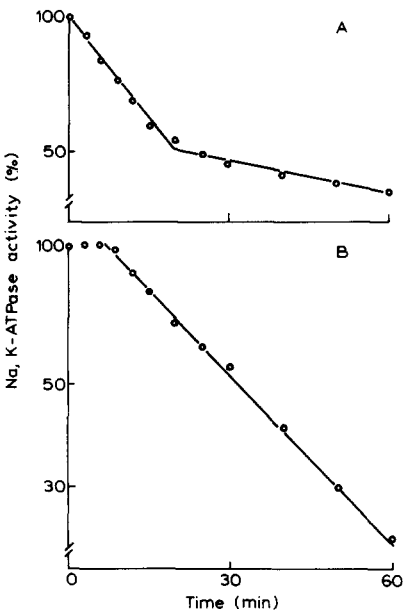


Fig. 5. Effects of 5 mM *p*-nitrophenyl phosphate alone (A) and in combination with 10 mM KCl (B) on trypsin inactivation of purified $(Na^+ + K^+)$-ATPase in the presence of 3 mM $MgCl_2$. The procedure was similar to that described in Fig. 4 except that the concentration of choline chloride was 150 – KCl (mM). Note: (i) the inactivation pattern in the presence of $MgCl_2$ plus *p*-nitrophenyl phosphate without KCl follows that described for enzyme in the E_1 conformation; (ii) addition of 10 mM KCl drastically changes the inactivation pattern which, after a delay of about 6 min, follows a single exponential.

simultaneously present. If *p*-nitrophenyl phosphate (PNPP) binds to the enzyme it might behave like inorganic phosphate leading to a $Mg \cdot E_2 \cdot PNPP$ form similar to the $Mg \cdot E_2 \cdot P_i$ complex [28] or it may preserve the E_1 state perhaps acting like ATP. Fig. 5A shows that the latter was the case (see also Ref. 29 for experiments on fluorescein-labeled $(Na^+ + K^+)$-ATPase). On the other hand, addition of 10 mM KCl to the forementioned ligands (Fig. 5B) drastically changed the digestion pattern: during the first six minutes there was no detectable change in $(Na^+ + K^+)$-ATPase activity, but from then on the decline followed a single exponential; this delay, not described before, was consistently seen in all experiments performed. The reason for this behavior is not known, but if we take the single exponential to represent an E_2 form we must conclude that this is the most abundant enzyme form under these conditions. However, and although a small fraction of the substrate will have been hydrolysed at the end of 6 min, in interpreting these results one must be cautious not to forget the possible effects of P_i and/or *p*-nitrophenol accumulation due to *p*-nitrophenyl phosphate hydrolysis during trypsin digestion. In experiments like that of Fig. 5A the P_i concentration at the end of the digestion period averaged about 0.2 mM whereas in those of Fig. 5B the average concentration was 1.5 mM. In the case of Fig. 5A, the fact that digestion behaved in the E_1 manner indicates that there was no P_i interference. On the other hand, an effect due to P_i accumulation cannot be completely ruled out in the experiment of Fig. 5B; nevertheless, due to the known *p*-nitrophenyl phosphate -P_i competition [6], it does not seem likely that the P_i concentration was high enough to bring most of the enzyme into the E_2 form. For these reasons, it is not unsafe to assume that in the presence of 3 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate and 10 mM KCl, E_2 is the predominant enzyme form; whether this form contains occluded K^+ remains an open question (see Discussion).

Discussion

One of the most common mechanism for substrate inhibition seems to be the binding of the substrate to a wrong enzyme form, or to a wrong site, leading to an enzyme form which is catalytically inactive or less active [30]. The protective action of K^+ (see also Refs. 6–8) and the potentiation due to Na^+, together with the ability of *p*-nitrophenyl phosphate to mimic ATP accelerating the release of Rb from the E_2(Rb) occluded complex, suggest that the wrong site at which *p*-nitrophenyl phosphate binds is the so called regulatory ATP site. If this is the case it becomes obvious that the substrate site for the phosphatase reaction cannot be the same at which ATP binds with low affinity as proposed [3]. The possibility that no clear line divides the ability of ATP and *p*-nitrophenyl phosphate to attach to each other's sites could account for the variable and some time contradictory reports on the effects of ATP on *p*-nitrophenyl phosphatase activity [7,10,22–24].

On the other hand, it could be also argued that the present results actually support the idea that

the phosphatase substrate site is that with low affinity for ATP. This view would be sustained by the mutual interference of ATP and *p*-nitrophenyl phosphate on phosphatase and $(Na^+ + K^+)$-ATPase activities [24] as well as for the following observation: (i) in the phosphatase reaction K^+ reduces the apparent affinity for *p*-nitrophenyl phosphate, and vice versa (Fig. 1, see also text), and (ii) *p*-nitrophenyl phosphate induces disocclusion of $E_2(K)$. If this interpretation were correct, substrate inhibition would have a mechanism different from that proposed above. However, the disoccluding ability of *p*-nitrophenyl phosphate is exerted by pushing the enzyme into a E_1 form. If the phosphatase substrate and ATP regulatory sites are the same, the implication is that once bound to the enzyme the substrate should take it away from, or prevent it to go to, the E_2 state. This is difficult to reconcile with the idea that E_2 is the catalytically active form in the phosphatase activity of $(Na^+ + K^+)$-ATPase [3,25,26].

In the presence of 3 mM MgCl and 5 mM *p*-nitrophenyl phosphate and in the absence of KCl, the rate of *p*-nitrophenyl phosphate hydrolysis in this preparation is 1% or less than that observed when 10 mM KCl is also included in the incubation solution (not shown). Fig. 5 shows that without K^+ the most abundant enzyme form is surely E_1, and that upon addition of 10 mM KCl the most abundant form is very likely of the E_2 type. This agrees with the idea that (i) a E_2 conformation is involved in the phosphatase activity of the $(Na^+ + K^+)$-ATPase, and (ii) one of the K^+ effects is precisely to bring the enzyme into that conformation. The possibility that this is not the only action of K^+ is given by the fact that in the presence of Na^+ and ATP external K^+ can take the enzyme into the E_2 state after dephosphorylation but is cannot stimulate the hydrolysis of *p*-nitrophenyl phosphate unless internal K^+ is present as well [12,31]; this was observed even under conditions where the intracellular K^+ concentration was much less than required to bring the enzyme into E_2 from the inside of the cell [31]. Whether the catalytically active form of the enzyme occludes K^+ cannot be asserted; however, if the binding of *p*-nitrophenyl phosphate to the substrate site produces a complex similar to $Mg \cdot E_2 \cdot P \cdot K$, K^+ occlusion is unlikely to take place.

If disocclusion follows the binding of the substrate to the wrong (ATP) site, taking the enzyme to a catalytically inactive form, it could explain the reduction in the apparent affinity for K^+ at increasing concentrations of *p*-nitrophenyl phosphate. At the same time, this could also account for the reduction in the apparent affinity for *p*-nitrophenyl phosphate when KCl is increased. The rationale is that at low KCl concentrations *p*-nitrophenyl phosphate begins to exert its inhibitory effect at relatively low concentrations and, as a result, the concentration of *p*-nitrophenyl phosphate giving half-maximal activity is much less than the concentration at which the substrate sites are half-filled. At high K^+ concentrations the inhibitory effect of *p*-nitrophenyl phosphate is much less and therefore the concentration giving half-maximal activity approaches the concentration at which the substrate sites are half filled.

Even acknowledging the uncertainties in the estimates, it is clear that the $K_{0.5}$ for *p*-nitrophenyl phosphate-induced disocclusion is much higher in experiments where the rate of Rb^+ release was measured (Table I) than in those performed under equilibrium conditions (Fig. 3). These results are to be expected for a case where the conversion of $E_2K \cdot PNPP$ to $E_1K \cdot PNPP$ is followed by the release of K^+ (Rb^+) which in turn displaces the equilibrium $E_2(K) + PNPP \rightleftharpoons E_2K \cdot PNPP$ to the right. In this mechanism the apparent affinities obtained from rate measurements are closer to the true affinities than those obtained from equilibrium determinations, which are much less than the real ones [32,33]. It is interesting that the $K_{0.5}$ of about 8 mM for *p*-nitrophenyl phosphate in disocclusion rate (Table I) is very close to the K_i of 9.5 mM for substrate inhibition in Fig. 2.

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