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Phosphatase activity of Na^+/K^+-ATPase. Enzyme conformations from ligands interactions and Rb occlusion experiments

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The present work compares the effects of several ligands (phosphatase substrates, $MgCl_2$, RbCl and inorganic phosphate) and temperature on the phosphatase activity and the E_2(Rb) occluded conformation of Na^+/K^+-ATPase. Cooling from 37 °C to 20 °C and 0 °C (hydrolysis experiments) or from 20 °C to 0 °C (occlusion experiments) had the following consequences: (i) dramatically reduced the V_{max} for *p*-nitrophenyl phosphate and acetyl phosphate hydrolysis but it produced little or no changes in the K_m for the substrates; (ii) led to a 5-fold drop in the K_m for the inorganic phosphate-induced di-occlusion of E_2(Rb); (iii) reduced the $K_{0.5}$ and curve sigmoidicity of the Rb-stimulated hydrolysis of *p*-nitrophenyl phosphate and acetyl phosphate and the Rb-promoted E_2(Rb) formation. At 20 °C, in the presence of 1 mM RbCl and no Mg^{2+}, acetyl phosphate did not affect E_2(Rb); with 3 mM $MgCl_2$, acetyl phosphate stimulated a release of Rb from E_2(Rb) both in the presence and absence of RbCl in the incubation mixture. As a function of acetyl phosphate concentration the K_m for Rb release was indistinguishable from the K_m found for stimulation of hydrolysis and enzyme phosphorylation under identical experimental conditions; in addition, the extrapolated di-occluded fraction corresponding to maximal hydrolysis was not different from 100%. These results indicate that although E_2(K) might be an intermediary in the phosphatase reaction, the most abundant enzyme conformation during phosphatase turnover is E_2 which has no K^+ occluded in it. The ligand interactions associated to phosphatase activity do not support an equivalence of this reaction with the dephosphorylation step in the $Na^+ + K^+$-dependent ATP hydrolysis; on the other hand, there are similarities with the reversible binding of inorganic phosphate in the presence of Mg^{2+} and K^+ ions.

Introduction

Na^+/K^+-ATPase preparations have always an associated phosphatase activity [1–3]. Because (i) acyl phosphate bonds are involved in the reaction, and (ii) Mg^{2+} and K^+ ions appear as the two essential activators, it has been proposed that that activity is equivalent to the dephosphorylation step in the $Na^+ + K^+$-dependent ATP hydrolysis [1–3]. However, the importance of the phosphatase reaction goes beyond that possibility and is related to the fact that it results from interactions of enzyme forms likely involved in the ATPase cycle with ligands definitely participating in that cycle. As such then, the understanding of the former could provide important information of the latter, which is still far from understood [1–3]. Attempts have been made to construct a phosphatase kinetic scheme [4–6]; this has been

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more difficult than for the $Na^+ + K^+$-dependent hydrolysis of ATP, due to the uncertainties about the actual enzyme forms participating, the intermediary steps involved and even the binding site for the substrate [7]. In the presence of K^+ and Mg^{2+} ions concentrations that are saturating for phosphatase activity the Na^+/K^+-ATPase is in a E_2 state [8–10] which occludes K^+ ($E_2(K)$) [11]; it is not surprising then that $E_2(K)$ was considered the essential and most abundant conformation during phosphatase turnover [1–3] (but see also Ref. 8). This idea has been challenged by recent experimental evidence indicating that, at least with *p*-nitrophenyl phosphate the major form during hydrolysis is an E_2 which does not seem to be occluding K^+ [10]. Although strongly suggestive, these experiments may not be completely convincing for two main reasons: (i) *p*-nitrophenyl phosphate is by itself a di-occluding agent (taking the enzyme into E_2) [7], and (ii) the relatively long incubation periods used (30 seconds) [10] allowed enough inorganic phosphate accumulation to account for at least some Rb^+ release from $E_2(Rb)$ [12]. We decided to reinvestigate the matter based on the interactions of Na^+/K^+-ATPase with ligands related to phosphatase activity taking advantage of two pieces of information: first, the fact that the poise of the E_1–E_2 equilibrium is strongly temperature dependent, with low temperatures favouring E_2 (see Ref. 12 for references), and second, the possibility of using acetyl phosphate as a substrate, a compound which unlike *p*-nitrophenyl phosphate does not affect the E_1–E_2 transition in the absence of magnesium (Ref. 13 and unpublished observations). In addition, we used a new mixing device that allowed much shorter phosphatase reaction times before Rb^+ occlusion was measured [14]. Some of the results presented in this paper have been communicated elsewhere [15].

Methods

Pig kidney Na^+/K^+-ATPase partially purified as proposed by Jørgensen [16] was used throughout this work. The specific activity varied between 13 and 20 units/mg. The enzyme suspension (3–4 mg/ml) was stored at −85 °C in a solution containing 25 mM imidazole (pH (20 °C) 7.5)/2 mM EDTA/10% sucrose. Before use the enzyme was washed twice with a similar solution without sucrose, and some times also without EDTA.

ATPase activity was assayed following the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ [7]; the labelling of $[\gamma\text{-}^{32}P]ATP$ was done as is Ref. 17 as modified in Ref. 18. The production of *p*-nitrophenol was determined as in Ref. 7 whereas the hydrolysis of acetyl phosphate was measured as in ref. 19 by the hydroxylamine method [20]. Total hydrolysis was always less than 10%. Before the assays the enzyme spent 5 min at 37 °C in media with and without 10^{-4} M ouabain after which it was equilibrated with the temperature of the incubation solution. When parallel Rb occlusion experiments were ran the enzyme was subjected to similar treatment but with no ouabain. Total hydrolysis was always less than 10%.

Occlusion of unphosphorylated Na^+/K^+-ATPase under equilibrium conditions was estimated as indicated in Ref. 7 incubating 0.1 mg of enzyme in 0.5 ml of solution containing 3 mM $MgCl_2$, 0.1 mM EGTA, 0.01 mM to 1 mM RbCl and enough Tris-HCl (pH 7.4 at the temperature of the experiment) to give a total ionic strength of 50 mM with and without 2 mM ADP. The equilibrated mixture was then passed through a Dowex 50-X8-400 resin column. The amount of occluded Rb was taken as the difference between the Rb present in the effluent without and with ADP. The release of ^{86}Rb from Rb-occluding enzyme was performed as described by Glynn et al. [14] using two syringes the contents of which were mixed immediately before the passage through a Dowex 50-X8-400 cation exchange column. One of the syringes (A) contained 0.5 ml of 0.1 mg enzyme protein equilibrated with 0.5 mM or 1 mM $^{86}RbCl$, 0.1 mM EGTA, variable Tris-HCl with and without $MgCl_2$. Syringe B had 0.7 ml of 0.1 mM EGTA, variable Tris-HCl with and without the addition of 0.5 mM or 1 mM RbCl and the other ligands to be tested (inorganic phosphate, acetyl phosphate, *p*-nitrophenyl phosphate or ADP) all at twice the desired final concentration. When the effects of these ligands were explored in the virtual absence of free Rb^+, 0.2 ml of Dowex resin were included at the bottom of syringe A [14]; in this way the concentration of free Rb reaching the mixing chamber was reduced to less than 0.5 μM.

The time elapsed between mixing and entrance to the final column was 0.3 s; for phosphatase reactions this meant an accumulation of inorganic phosphate of less than 5 μM at 20 °C.

Enzyme phosphorylation from acetyl[^{32}P]phosphate was carried out incubating 0.2 mg of the enzyme at 20 °C in 0.5 ml of a solution containing 1 mM RbCl, 3 mM $MgCl_2$, 1 to 5 mM acetyl phosphate and enough concentration of Tris-HCl (pH (20 °C) 7.4) to give a total ionic strength of 50 mM. After 10 s the reaction was stopped by the addition of 20% trichloroacetic acid and 50 mM inorganic phosphate (all final concentrations). The mixture was allowed to stand for 15 min at 0 °C and the denatured protein was collected in a Whatman GF/F glass fiber filter; the filter was then washed with 30 ml of 5% trichloroacetic acid/10 mM inorganic phosphate and counted in a liquid scintillation counter using a toluene based scintillator. The synthesis of acetyl [^{32}P]phosphate was done as proposed by Stadtman [20] with [^{32}P]P_i purified as in Ref. 21. Phosphorylation with [γ-^{32}P]ATP was done in a similar way except that incubation time was 5 s, the nucleotide concentration 10 μM, total amount of enzyme 0.04 mg and the stopping solution contained in addition 2 mM unlabelled ATP.

Protein was routinely determined by Lowry et al. [22] using bovine serum albumin as standard; in Rb^+ occlusion experiments the Peterson's [23] modification of Lowry et al. was used.

All solutions were made with de-ionized bi-distilled water. NaCl and RbCl were spectrometric grade; the other chemicals were reagent grade. ATP, acetyl phosphate, *p*-nitrophenyl phosphate, Dowex and ouabain were from Sigma Chemical Co., U.S.A. [^{32}P]P_i was purchased from the Comisión Nacional de Energía Atómica of Argentina. A pH of 7.4 and a total ionic strength of 50 mM were maintained in all experiments by including enough Tris-HCl. Additional information on solutions composition in the individual experiments is given in the table and figure legends.

Counting was performed in a Beckman liquid scintillation counter with automatic quenching correction; when possible, counting was long enough to give a standard error of about 1%.

Unless otherwise stated each experiment was performed in triplicate and it was repeated at least twice. The curve fitting of the experimental points was executed with a non-linear regression computer program.

Results

Effects of temperature on the enzyme–ligand interactions during phosphatase activity

Table I summarizes the substrates kinetic parameters of ouabain-sensitive *p*-nitrophenyl phosphate and acetyl phosphate hydrolysis measured at 37 °C, 20 °C and 0 °C. $MgCl_2$ and RbCl (replacing KCl) were maintained at 3 mM and 10 mM, respectively. As it can be seen, lowering the temperature over that wide range slightly affected the K_m for the substrates (about 47% increase for *p*-nitrophenyl phosphate and 35% decrease for

TABLE I

TEMPERATURE EFFECTS ON SUBSTRATE KINETIC PARAMETERS OF OUABAIN-SENSITIVE *p*-NITROPHENYLPHOSPHATASE AND ACETYLPHOSPHATASE ACTIVITIES OF Na^+/K^+-ATPase

Na^+/K^+-ATPase was incubated in solutions containing 10 mM RbCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM to 5 mM *p*-nitrophenyl phosphate or acetyl phosphate and enough Tris-HCl to give a total ionic strength of 50 mM. The K_m values were calculated fitting the experimental points to hyperbolic functions. Each entry is the mean ± S.E. of three different experiments. The pH was 7.4 at all temperatures. See Methods for details.

Temp (°C)	*p*-Nitrophenylphosphatase activity		Acetylphosphatase activity	
	K_m (mM)	V_{max} (μmol·mg^{-1}·min^{-1})	K_m (mM)	V_{max} (μmol·mg^{-1}·min^{-1})
37	2.25 ± 0.20	4.30 ± 0.02	2.01 ± 0.25	16.04 ± 0.84
20	2.73 ± 0.14	1.83 ± 0.03	1.80 ± 0.19	5.50 ± 0.26
0	3.32 ± 0.30	0.23 ± 0.01	1.34 ± 0.11	0.44 ± 0.02

TABLE II

EFFECTS OF TEMPERATURE ON THE $K_{0.5}$ AND HILL COEFFICIENTS FOR Rb^+ STIMULATION OF OUABAIN-SENSITIVE *p*-NITROPHENYLPHOSPHATASE AND ACETYLPHOSPHATASE ACTIVITIES OF Na^+/K^+-ATPase

Na^+/K^+-ATPase was incubated in solutions containing 3 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate or acetyl phosphate, 0.1 mM EGTA, 0 mM to 10 mM RbCl and enough Tris-HCl to give a total ionic strength of 50 mM. The general procedure was similar to that described in the legend to Table I. The values of the listed parameters were calculated fitting the experimental points to the Hill equation. Each entry is the mean ± S.E. of three different experiments. See Methods for details.

Temp. (°C)	*p*-Nitrophenylphosphatase activity		Acetylphosphatase activity	
	$K_{0.5}$ (mM)	n_H	$K_{0.5}$ (mM)	n_H
37	3.03 ± 0.06	2.27 ± 0.14	1.21 ± 0.09	2.65 ± 0.22
20	0.75 ± 0.01	1.77 ± 0.11	0.39 ± 0.10	1.84 ± 0.03
0	0.18 ± 0.01	1.21 ± 0.09	0.09 ± 0.02	1.43 ± 0.07

acetyl phosphate), whereas it produced a noticeable reduction in the maximal rates of hydrolysis. From the V_{max} values the calculated activation energies are roughly 9–11 kcal/mol between 37 °C and 20 °C and 16–20 kcal/mol between 20 °C and 0 °C.

Table II shows the kinetic parameters for Rb^+ stimulation of phosphatase activity, also measured at 37 °C, 20 °C and 0 °C, in the presence of 3 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate or acetyl phosphate. With both substrates the apparent affinity for RbCl markedly increased upon cooling. Comparing 37 °C with 0 °C, the $K_{0.5}$ values underwent a 14–17-fold reduction while the Hill coefficients changed from 2.3–2.6 to 1.2–1.4.

Effects of temperature on the enzyme–ligand interactions leading to occlusion and release of occluded rubidium

The following group of experiments explored the influence of temperature on the interactions of two ligands with enzyme forms likely related to phosphatase activity: that of Rb^+ with E_1, leading to $E_2(Rb)$ and that of inorganic phosphate with $E_2(Rb)$ producing E_2P and releasing Rb^+. These results are summarized in Table III. In the presence of 3 mM $MgCl_2$ the ability of RbCl to induce Rb^+ occlusion of unphosphorylated enzyme under equilibrium conditions is tremendously facilitated by lowering the temperature from 20 °C to 0 °C: i.e. an almost 6-fold reduction in the $K_{0.5}$ for RbCl and a change in the Hill coefficient from about 2.2 to a value not different from 1.0 (First two columns in Table III). On the other hand, for an identical temperature drop the K_m for the

TABLE III

EFFECTS OF TEMPERATURE ON THE $K_{0.5}$ AND HILL COEFFICIENT FOR Rb^+ ON Rb^+ OCCLUSION AT EQUILIBRIUM AND THE K_m FOR INORGANIC PHOSPHATE STIMULATED RELEASE OF OCCLUDED Rb^+ IN Na^+/K^+-ATPase

Occlusion of Rb^+ ions under equilibrium was measured as described in Methods and in Ref. 7; RbCl concentrations ranged from 0.01 mM to 1 mM. The concentration of $MgCl_2$ was 3 mM and there was enough Tris-HCl to give a total ionic strength of 50 mM. The effects of inorganic phosphate on the release of Rb from Rb^+-occluding Na^+/K^+-ATPase were explored as indicated in Ref. 14 using two syringes, a mixing chamber and a cation exchange resin column. The syringe containing the occluded $E_2(Rb)$ enzyme also had a 0.2 ml resin column in order to remove external Rb^+ before exposure to inorganic phosphate. The experimental points were fitted to Hill equations (effects of RbCl on Rb^+ occlusion) or hyperbolic equations (effects of inorganic phosphate on Rb^+ release). Each entry is the mean ± S.E. of three different experiments. For details see Methods.

Temp. (°C)	Rubidium chloride		Inorganic phosphate
	$K_{0.5}$ (mM)	n_H	K_m (mM)
20	0.080 ± 0.010	2.22 ± 0.04	0.096 ± 0.010
0	0.014 ± 0.002	1.11 ± 0.15	0.012 ± 0.004

inorganic phosphate induced Rb^+ release from $E_2(Rb)$ (in the presence of 1 mM $MgCl_2$ and in the absence of Rb) was also reduced by around 6-fold (last column of Table III). Similar result were found in the presence of 1 mM RbCl (not shown).

Effects of acetyl phosphate on Rb^+ occlusion during phosphatase turnover

A possible relationship between phosphatase

turnover and the occluded E_2(Rb) was further investigated comparing steady-state measurements of acetyl phosphate hydrolysis and Rb^+ occlusion as a function of acetyl phosphate concentration at 20 °C. Ouabain-sensitive hydrolysis was determined after 20 min incubation. On the other hand, in order to avoid accumulation of inorganic phosphate in occlusion experiments E_2(Rb) ATPase interacted with acetyl phosphate for 0.3 s; this was accomplished using two syringes and a mixing chamber as described in Methods. In the absence of ionized Mg^{2+}, acetyl phosphate did not have any effect on Rb^+ release; therefore, the values reported in the figure correspond to the differences between acetyl phosphate and ADP both in the presence of $MgCl_2$ (see Methods).

Four aspects of these results, illustrated in Figs. 1A and 1B, must be remarked: (i) acetyl phosphate is hydrolyzed by the enzyme and induces di-occlusion of Rb^+ only in the presence of $MgCl_2$; (ii) the K_m for the two forementioned effects are indistinguishable from each other (1.35 ± 0.05 mM and 1.2 ± 0.15 mM, respectively); (iii) the fractional di-occlusion observed above 1 mM acetyl phosphate is larger than 50%, a maximum value to be expected if it were due to phosphorylation by inorganic phosphate (Refs. 10, 14; personal results not shown); (iv) the maximal Rb^+ release extrapolated to maximal rate of hydrolysis was 94 ± 4.9%; value which is not different from 100%.

In another group of experiments we used *p*-nitrophenyl phosphate instead of acetyl phosphate. As reported before [7] we found Rb release induced by *p*-nitrophenyl phosphate in the absence and presence of $MgCl_2$, but that release was always significantly larger with $MgCl_2$ in the incubation solution (results not shown).

The set up used in these experiments gave us the unique opportunity to explore the effects of acetyl phosphate on Rb occlusion in the absence of RbCl in the reaction mixture, a condition of obvious relevance to phosphatase reaction. To this aim [14] we included a 0.2 ml Dowex 50-X8-400 column in the syringe containing the enzyme equilibrated with $^{86}RbCl$. In this way E_2(Rb) Na^+/K^+-ATPase free from external Rb^+ could be exposed to the effects of the desired ligands, including RbCl, coming from the other syringe. The results of these experiments are summarized in Table IV. They clearly indicate that in the presence of $MgCl_2$ acetyl phosphate was able to induce di-occlusion of E_2(Rb) both in the presence and virtual absence of free Rb^+ in the reaction mixture; the fraction of Rb^+ released was similar without (70%) and with (65%) RbCl. As seen before, no effect of acetyl phosphate was observed without $MgCl_2$ in the media.

TABLE IV

EFFECTS OF ACETYL PHOSPHATE ON Rb RELEASE FROM Rb^+-OCCLUDING Na^+/K^+-ATPase IN THE PRESENCE AND ABSENCE OF Rb^+ AND/OR Mg^{2+} IN THE INCUBATION SOLUTION

The bottom of syringe A consisted of a 0.2 ml Dowex cation exchange resin column equilibrated with solution A without RbCl. On top of the resin there was 0.5 ml of solution A having, in addition to what is indicated in the table, 0.1 mg of Na^+/K^+-ATPase protein, 1 mM $^{86}RbCl$, 0.1 mM EGTA and Tris-HCl (pH (20 °C) 7.4). Syringe B contained 0.7 ml of solution (B) with what is shown in the table plus 0.1 mM EGTA, Tris-HCl (pH (20 °C) 7.4) and 0 mM or 4 mM ADP. Total ionic strength was adjusted to 50 mM with Tris-HCl. The temperature was 20 °C. Each entry is the mean ± S.E. of triplicate determinations.

Syringe A	Syringe B			Rb^+ in effluent (Total − ADP)	
$MgCl_2$ (mM)	$MgCl_2$ (mM)	RbCl (mM)	Acetyl phosphate (mM)	nmol/mg	%
0	0	2	0	3.92 ± 0.23	100
0	0	2	10	3.90 ± 0.18	99
3	3	2	0	3.85 ± 0.16	100
3	3	2	10	1.35 ± 0.13	35
0	0	0	0	3.74 ± 0.20	100
0	0	0	10	3.50 ± 0.21	94
3	3	0	0	3.76 ± 0.13	100
3	3	0	10	1.08 ± 0.10	29

Steady-state levels of enzyme phosphorylation during phosphatase activity

It has been stablished that enzyme phosphorylation from inorganic phosphate leads to release of occluded Rb^+ from E_2(Rb) while the conformation of the enzyme still remained in E_2 [10,14,24]. Considering the proposed relationship between phosphatase activity and the dephosphorylation reaction in the ATPase cycle [1–3] it seemed important to investigate if (i) under phosphatase turnover using acetyl phosphate phosphoenzyme formation took place, and (ii) if that was the case,

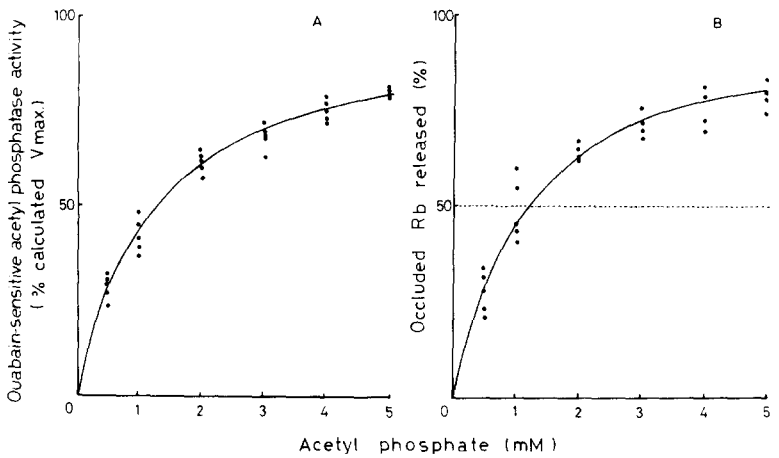

Fig. 1. Effects of different concentrations of acetyl phosphate on the ouabain-sensitive phosphatase activity (A) and the release of occluded Rb from E_2(Rb) (B) in Na^+/K^+-ATPase incubated in the presence of 1 mM RbCl, 3 mM $MgCl_2$, 0.1 mM EGTA and enough Tris-HCl (pH (20 °C) 7.4) to give a total ionic strength of 50 mM. The temperature was 20 °C and pH 7.4. Phosphatase activity was estimated as described in Methods after 20 min incubation. The loss of Rb from E_2(Rb) Na^+/K^+-ATPase was determined with the aid of two syringes, a mixing device and a cation exchange (Dowex) resin column as described in Methods and Ref. 14. The reaction time in this case was 0.3 s. Temperature was 20 °C. Each point in the figure corresponds to duplicate (A) or triplicate (B) determinations of different experiments. The lines through the points are the best fit to hyperbolic functions. Phosphatase activity is expressed as percentage of the maximal calculated rate. The horizontal dotted line in (B) is the average maximal di-occlusion obtained when inorganic phosphate (1 M) was used instead of acetyl phosphate.

what fraction of the total enzyme remained phosphorylated under steady-state conditions. Phosphorylation experiments were carried out incubating the enzyme for 10 s at 20 °C in the same solutions where phosphatase activity and release

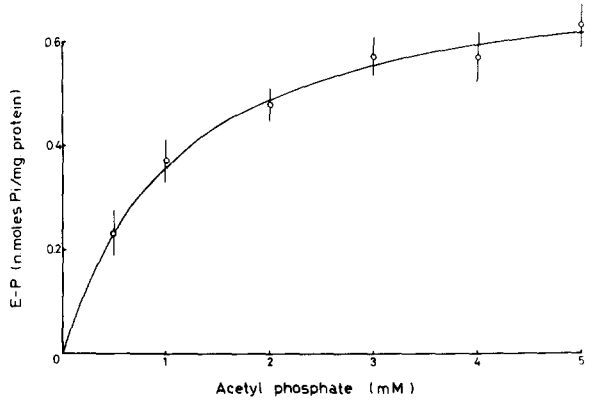

Fig. 2. Effects of acetyl phosphate concentrations on the steady-state phosphorylation levels of Na^+/K^+-ATPase incubated in the presence of 1 mM RbCl, 3 mM $MgCl_2$, 0.1 mM EGTA and enough Tris-HCl (pH (20 °C) 7.4) to give a total ionic strength of 50 mM. Each point is the mean ± S.E. of triplicate determinations. The curve through the points is the best fit to an hyperbolic function with a K_m of 1.11 ± 0.15 mM and a maximal phosphorylation of 0.73 ± 0.04 μmol P_i per mg protein.

of occluded Rb were measured (see Fig. 1). The results of one of these experiments, illustrated in Fig. 2, show that under the conditions explored there is a steady-state level of an acid stable phosphoenzyme which increases together with the concentration of acetyl phosphate. The experimental points fitted with an hyperbolic function gave a K_m for acetyl phosphate of 1.11 ± 0.15 mM and a maximal amount of phosphoenzyme of 0.73 ± 0.04 nmol per mg protein. When checked, similar levels of phosphorylation were found after 1 and 30 s incubation. In addition, under these ionic conditions there was no detectable enzyme phosphorylation when acetyl phosphate was replaced by 10 μM [γ-^{32}P]ATP. Furthermore, the maximal Na^+-dependent phosphorylation from ATP in this preparation was 1.93 ± 0.07 nmol/mg protein.

Discussion

There are two interconnected aspects that must be contemplated when analyzing the results presented in this paper: (1) their specific relevance to phosphatase activity, and (2) their contribution to the understanding of the proper function of the enzyme, the hydrolysis of ATP.

Phosphatase reaction

The almost identical temperature dependence of Rb stimulation of phosphatase activity and E_2(Rb) formation is consistent with the notion that an E_2 form is essential to phosphatase reaction [10,12], but it might also imply that somehow E_2(K) is involved. On the other hand, the experiments in Fig. 1 indicate that the most abundant form during phosphatase turnover does not occlude K^+; i.e. to maximal velocity of hydrolysis corresponds a Rb^+ release of, or close to, 100%. Then, how does E_2(K) fit in a kinetic scheme for phosphatase? Occlusion as used here is an operational definition based on the ADP-sensitive Rb trapped in the enzyme after 0.7 s exposure to a cation exchange resin. If E_2(K) is indeed a phosphatase intermediary, the results of Fig. 1 could be accounted for in at least two ways: (1) an occluded state exists all the time, but the stability of this enzyme-Rb complex is smaller than that of the classical E_2(Rb); (2) the occluded state as defined here only exists during a small fraction of the reaction cycle. The second explanation implicity assumes an alternation of occluded non-occluded states during phosphatase turnover; that alternation could be based on E_2(K)–E_1K or E_2(K)–E_2K transitions. The existence of a transitory E_1K is unlikely for under these conditions (absence of ATP and Na) oligomycin, which binds only to E_1 does not affect phosphatase activity [1–3]. On the other hand, E_2K could result from the combination of the ternary complex Mg·E_2(K) not only with inorganic phosphate but with the substrate and the other product, *p*-nitrophenol, as well (note that in steady state, at 20 °C and maximal rate of acetyl phosphate hydrolysis, about 40% of the available enzyme is phosphorylated (see also Refs. 5 and 25)); this hypothesis has the additional attraction that it could explain the fractional di-occlusion larger than the 50% expected in E-P formation from inorganic phosphate (Refs. 14, 24, 25, 26, this work) if under these conditions K release is random (see below).

Another point calling for comments is the observation that, provided $MgCl_2$ is present, acetyl phosphate can induce Rb release from E_2(Rb) even in the virtual absence of free Rb. A likely explanation is that phosphatase activity can take place without K^+ in the media as long as E_2(K) exists (under our experimental conditions this would have meant the production of one single reaction cycle). In this regard we have observed enzyme phosphorylation when E_2(Rb) was exposed for 0.25 s to acetyl phosphate and $MgCl_2$ in the absence of RbCl at 0 °C and 20 °C; unfortunately these results are complicated by the fact that similar levels of E-P formation can be seen without any monovalent cation (not shown). The possibility that in the absence of free Rb^+, E_2(Rb) can sustain one cycle of phosphatase activity does not necessarily dispute the existence of more than one type of K^+ stimulatory sites for this reaction [5,7]; this is so because it might be that the duration of that cycle is not the same with and without free Rb^+ in the reaction mixture.

Na^+/K^+-ATPase reaction

There seems to be no firm basis to support the notion that phosphatase and K^+-stimulated dephosphorylation in the Na^+/K^+-ATPase have much in common; actually, the fact that K^+ stimulates E-P breakdown from external sites [1–3] whereas full phosphatase activity can be obtained with K^+ acting intracellularly in the complete absence of external K^+ [27,28] is enough evidence against it. On the other hand, it might be useful to look into another part of the ATPase cycle, that involving reversible binding of inorganic phosphate and its associated K^+–K^+ exchange.

Considering the temperature effects on the K_m for the substrates there is a marked difference in sensitivity between the phosphatase substrates on the one side and the inorganic phosphate induced di-occlusion on the other (Tables I and III). There is no obvious reason for this behaviour; possible explanations are that phosphorylation by inorganic phosphate [29] is a much simpler reaction that phosphatase activity [4–6] or that, despite the competition seen between inorganic phosphate and *p*-nitrophenyl phosphate [4] the actual binding sites of both compounds are not the same. It should be stressed here that these results cannot be attributed to a reduction in the rate of Rb^+ release at low temperatures for it can be easily shown that that would tend to increase, not decrease the K_m for inorganic phosphate.

The simultaneous presence of inorganic phosphate, ATP (or an analogue which does no have to

be hydrolyzable) and Mg^{2+} are essential for K^+–K^+ exchange through the Na^+/K^+ pump [30]. This is so because upon enzyme phosphorylation occluded K^+ ions, which were initially bound to inside sites, are release at the external side of the membrane; this process is thought to be ordered [14,24] to account for the observation that in the presence of enough K^+ in the mixture, only 50% of the occluded ions are lost following phosphorylation by inorganic phosphate (Refs. 14, 24, 26, and this work). The inward K^+ translocation requires ATP to speed up the $E_2(K)$–E_1K transformation followed by the discharge of K^+ on the inside. On the other hand, all attempts to demonstrate a cation translocation associated to phosphatase reaction have failed (see Refs. 1–3 for references); this is extremely important because *p*-nitrophenyl phosphate is by itself able to accelerate the E_2–E_1 conformational change [7]. This can be explained if, as proposed above, the E_2–E_1 transition does not take place during phosphatase activity. One can then visualize the phosphatase reaction as resembling the reversible binding of inorganic phosphate in the absence of ATP [29]; in this case, if the enzyme is phosphorylated without K^+, these ions do not affect dephosphorylation, whereas the previous existence of $Mg \cdot E_2(K)$ accelerates both the rates of phosphorylation and dephosphorylation [29]. It is conceivable that in the phosphatase reaction the ternary complex $Mg \cdot E_2(K)$ must be formed before the substrate can get into the catalytic site. The main problem remaining with this idea is the total di-occlusion during phosphatase activity compared with half di-occlusion in phosphorylation by inorganic phosphate. If K^+ release during phosphatase turnover is solely consequence of E-P formation one is forced to conclude that in this case K^+ ions leave the enzyme in a random fashion; but the molecular mechanism underlying this differential behaviour remains unknown.

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