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S.E. Faraj, W.M. Valsecchi, N.T. Cerf, N.U. Fedosova, R.C. Rossi, M.R. Montes

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The interaction of Na^+, K^+, and phosphate with the gastric H,K-ATPase. Kinetics of *E*1–*E*2 conformational changes assessed by eosin fluorescence measurements.

SE. Faraj[a,b,†], WM. Valsecchi[a,b,†], NT. Cerf[a,b], NU. Fedosova[c], RC. Rossi[a,b], MR. Montes[a,b,*]

a Universidad de Buenos Aires. Facultad de Farmacia y Bioquímica. Departamento de Química Biológica. Buenos Aires, Argentina. ***b*** Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – Universidad de Buenos Aires. Instituto de Química y Fisicoquímica Biológicas "Prof. Alejandro C. Paladini" (IQUIFIB). Buenos Aires, Argentina. ***c*** Department of Biomedicine, Aarhus University, Aarhus, Denmark.

* To whom correspondence should be addressed: Mónica R. Montes, Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina; mmontes@qb.ffyb.uba.ar; Tel. (+54) 011 4964-8289; Fax. (+54) 011 4962-5457.

† These authors contributed equally to this work.

ABSTRACT

H,K-ATPase and Na,K-ATPase show the highest degree of sequence similarity among all other members of the P-type ATPases family. To explore their common features in terms of ligand binding, we evaluated conformational transitions due to the binding of Na^+, K^+ and Pi in the H,K-ATPase, and compared the results with those obtained for the Na,K-ATPase. This work shows that eosin fluorescence time courses provide a reasonably precise method to study the kinetics of the *E*1−*E*2 conformational changes in the H,K-ATPase. We found that, although Na^+ shifts the equilibrium toward the *E*1 conformation and seems to compete with H^+ in ATPase activity assays, it was neither possible to isolate a Na^+-occluded state, nor to reveal an influx of Na^+ related to H,K-ATPase activity. The high rate of the *E*2K→*E*1 transition found for the H,K-ATPase, which is not compatible with the presence of a K^+-occluded form, agrees with the negligible level of occluded Rb^+ (used as a K^+ congener) found in the absence of added ligands. The use of vanadate and fluorinated metals to induce *E*2P-like states increased the level of occluded Rb^+ and suggests that—during dephosphorylation—the probability of K^+ to remain occluded increases from the *E*2P-ground to the *E*2P-product state. From kinetic experiments we found an unexpected increase in the values of k_{obs} for *E*2P formation with [Pi]; consequently, to obey the Albers-Post model, the binding of Pi to the *E*2 state cannot be a rapid-equilibrium reaction.

Keywords: H,K-ATPase, Na,K-ATPase, conformational changes, enzyme mechanism, enzyme kinetics, ligand binding

1. Introduction

P-type ATPases constitute a group of membrane proteins that couple the energy released from ATP hydrolysis to the transmembrane translocation of molecules. Ion-transporting members of the P-type ATPase family maintain significant transmembrane gradients of their ligands, which are critical for membrane potential, secondary active transport of nutrients and volume regulation in eukaryotic cells [1, 2]. Thus, gastric H,K-ATPase actively extrudes H^+ from the cytoplasm (pH≈7) into the lumen and creates an extracellular pH as low as 1. Likewise, Na,K-ATPase maintains a significant electrochemical gradient for Na^+. Both enzymes couple H^+ or Na^+ extrusion to the uptake of K^+ and are therefore anticipated to have a high degree of structural and functional similarity, as confirmed by, for example, the presence of beta subunits and the general applicability of the Albers-Post model. The model considers two main conformations of the enzymes, *E*1 and *E*2, both in the auto-phosphorylated and non-phosphorylated forms (Figure 1). The ion binding sites alternate between being open toward the cytoplasmic and extracellular sides. These transitions couple the scalar processes of ATP binding, phosphorylation, and dephosphorylation to the vectorial transport of cations [2].

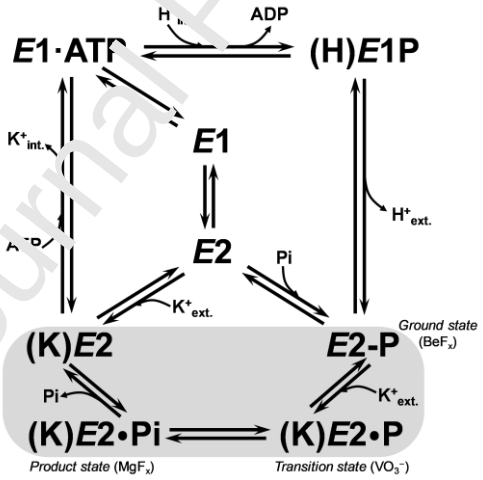

Figure 1. A simplified version of the Albers-Post model for the functioning of H,K-ATPase. The shaded area highlights the consecutive states involved in the dephosphorylation reaction due to K^+ binding.

The cycles of H,K-ATPase and Na,K-ATPase start with the binding of H^+ or Na^+ on the intracellular side followed by the most energy-consuming process of outward transport of the corresponding ion. Yet, ions specificity of these pumps is not absolute [3-5]. An alternative cycling mode in the absence of Na^+ has been described for the Na,K-ATPase [6, 7], and has led to the proposal of a "0 ATPase", where exchange may occur between H^+ and K^+. On the other hand, for the H,K-ATPase, Rabon *et al.* [4] claimed that Na^+ shifts the equilibrium to *E*1, and Polvani *et al.*

[5] suggested that Na^+ can replace H^+ during the transport cycle. To understand the role of Na^+ as a substitute for H^+, in this study we analyze the effect of Na^+ on the H,K-ATPase regarding conformational changes, cation occlusion and ATPase activity.

For both, H,K-ATPase and Na,K-ATPase, phosphorylation opens the cation sites from the extracellular side. Binding of K^+ activates dephosphorylation (Figure 1), a process including a sequence of reactions from the *E*2P-ground to the *E*2P-product state where Pi and K^+ interactions with the enzyme change in consort. Here we analyze these relationships by following K^+ (Rb^+) occlusion in various *E*2P-like states stabilized by metal-fluoride compounds and vanadate.

Given the high resemblance of the Na,K-ATPase and the H,K-ATPase, and the fact that eosin fluorescence was highly useful for describing ion interactions with the non-phosphorylated forms of the Na,K-ATPase, we applied this dye to study H,K-ATPase. Unlike previous studies with fluorescein-labeled protein [4, 8], where the enzyme activity is abolished, eosin binds non-covalently to the protein, allowing, as far as we know, the first measurements of the kinetics of conformational changes of native (non-labeled) H,K-ATPase. We monitored the equilibrium distribution and the rate of transitions between *E*1 (high signal) and *E*2 (low signal) states of the H,K-ATPase induced by Na^+, K^+ and Pi.

2. Materials and Methods

2.1. Enzyme preparations

H,K-ATPase-enriched membrane vesicles were prepared as described by Sachs *et al.* [9], with some modifications as described elsewhere [10]. The preparation was essentially free from contamination with Na,K-ATPase as shown by the absence of ouabain-sensitive activity. The specific ATPase activity at the time of preparation was 55−65 μmol of Pi h^{-1} (mg protein)$^{-1}$, measured in reaction media containing 25 μg protein ml^{-1}, 12.5 μg alamethicin ml^{-1} (incubated with the enzyme for 20 min before the assay), 2.2 mM ATP, 4 mM $MgCl_2$ and 10 mM KCl, in 25 mM imidazole–HCl, pH 7.4 at 25 °C. The maximum number of nucleotide binding sites in the preparation was 1.4 nmol per mg of protein. Na,K-ATPase was partially purified from pig kidney according to Klodos *et al.* [11]. The specific activity at the time of preparation was 230–250 μmol of Pi h^{-1} (mg protein)$^{-1}$ measured under optimal conditions (150 mM NaCl, 20 mM KCl, 3 mM ATP, and 4 mM $MgCl_2$ in 25 mM imidazole–HCl, pH 7.4 at

25 °C). The maximum number of nucleotide binding sites in the preparations used in this work was 2.4 nmol of sites per mg of protein.

2.2. Reagents

Rb^+ was used as a congener of K^+. [^{86}Rb]RbCl, [^{22}Na]NaCl, and [γ-^{32}P]ATP were obtained from *Perkin-Elmer Life Sciences* (USA). The fluorescent probe eosin-Y (eosin), alamethicin, phosphate acid, $BeSO_4$, NaF, Na_3VO_4 and ATP were from *Sigma Chemical Co* (USA). NaF was passed through the AG MP50 resin (*BioRad*, USA) equilibrated with imidazole to obtain imidazolium fluoride (ImF). Epigallocatechin-3-gallate (EGCg) was obtained from *Aktin Chemicals* (China). All other reagents were of analytical grade.

2.3. Reaction conditions

Unless otherwise stated, incubations were performed at 25 ºC in media containing 25 mM imidazole-HCl (pH 7.4 at 25 ºC) and 0.25 mM EDTA. For experiments performed at pH 8.4, 25 mM Tris-HCl (pKa 8.1) was used. H,K-ATPase preparations were permeabilized with the channel-forming peptide alamethicin, which was dissolved in 50 % v/v ethanol to a concentration of 10 mg ml^{-1}. The final concentration of ethanol never exceeded 0.5 % in volume. Maximal activation of the ATPase by alamethicin (approximately 75 %) was reached at a mass relation (alamethicin/protein) of 0.45 and remained constant from 30 to (at least) 80 minutes of incubation time. The concentration of BeF_3^- was calculated from the total concentration of beryllium and fluoride according to Mesmer and Baes [12].

2.4. ATPase activity

H,K-ATPase activity was assayed as the release of ^{32}Pi from [γ-^{32}P]ATP at 25 ºC. Reactions were performed using about 5 µg of protein in the presence of 2.2 mM ATP, 4 mM $MgCl_2$ and 10 mM KCl. We employed the method described by Schwarzbaum *et al.* [13] to quantitatively extract the Pi present in the media.

2.5. Conformational changes

Conformational states were studied by measuring the eosin fluorescence signal which is high for states in *E*1 and is low for states in *E*2 [14, 15]. Note that since eosin is used to monitor the formation/break-down of the *E*1 conformation, it cannot discriminate between different *E*2 states. Equilibrium measurements were carried out in a Jasco FP-6500 spectrofluorometer. The excitation wavelength was 518 nm, with a band-pass of 3 nm, and emission spectra were collected in the 525−570 nm range. It is worth noting that for cation concentrations above 20 mM, we

observed a decrease of eosin fluorescence as also described by Helmich-de Jong *et al.* [16], which is probably related to the effect of ionic strength on the affinity for eosin [17].

Measurements of the time course of fluorescence were performed with a stopped-flow reaction analyzer (SX-18MV, *Applied Photophysics*). For each experimental condition, between four and six time traces (each one of 1000 data points) were averaged. The excitation wavelength was 518 nm, and emitted light was filtered through an OG550 filter (*Schott Advanced Optics*).

2.6. Rb^+ and Na^+ occlusion and uptake assays

$^{86}Rb^+$ and $^{22}Na^+$ occlusion (tight binding) and $^{22}Na^+$ uptake (retention inside vesicles) were measured according to Rossi *et al.* [18]. Briefly, reactions were carried out in a rapid-mixing apparatus (SFM4 from *Bio-Logic*, France) connected to a quenching and washing chamber. The media, containing all reacting species, was sent through a 0.8 µm filter (*Millipore*). The filter was thoroughly washed with an ice-cold buffer. Next, the retained radioactivity was measured. Blanks were obtained using thermally inactivated protein (50 min at 60 °C). Equilibrium occlusion of cations was attained by incubating enzyme during at least 20 min. In this case, treatment with the ionophore alamethicin prevented $^{86}Rb^+$ and $^{22}Na^+$ accumulation inside the vesicles (see Figure S1 in *Supplementary Material*), so that only occluded ions (tightly bound within the transmembrane segments) were quantified [10]. Time courses of Na^+ uptake were measured after mixing one volume of enzyme suspension (in the absence of alamethicin) with one volume of a solution containing $^{22}Na^+$ and incubated at 25 ºC for varying lengths of time.

2.7. Data analysis

The equations were fitted to the experimental data by a nonlinear regression procedure using *OriginPro 2017*. Parameters are expressed as value ± standard error (S.E.).

3. Results and Discussion

3.1. E1 and E2 states followed by eosin fluorescence

Eosin is a fluorescent dye that raises its signal upon binding with high affinity to the ATP-site of certain ATPases. It has been extensively used for equilibrium and transient experiments in the Na,K-ATPase [14, 15, 19], but only in equilibrium assays in the H,K-ATPase [16, 20]. Here we used this probe to quantify the conformational states *E*1 (high affinity) and *E*2 (low affinity) and the velocity of the *E*1−*E*2 transitions during elementary steps of the

ATPase cycle. To corroborate that eosin binding is much faster than the $E1-E2$ transition, we firstly assessed the kinetics of eosin binding to the H,K-ATPase (Figure 2). Na,K-ATPase was used as a control. For both enzymes, a very good description of the time courses could be achieved by an exponential function of time:

$$F(t) = \Delta F \left(1 - e^{-k_{obs}\, t}\right) + F_0 \qquad \text{Eq. 1}$$

where F_0 is the initial fluorescence value, ΔF is the total fluorescence change and k_{obs} is the observed rate constant of the process. The best fitting of Eq. 1 to the data in Figure 2 (Panels A and B) shows that eosin binding to the H,K-ATPase takes place with a k_{obs} of $52.9 \pm 1.1\ s^{-1}$, which is almost identical to what we ($56.4 \pm 0.3\ s^{-1}$) and others [19] obtained for the Na,K-ATPase. These values show that the kinetics of eosin binding is not a rate-limiting step to measure the $E1-E2$ transition.

In equilibrium assays, where the H,K-ATPase was incubated with eosin (Figure 2C), the addition of Mg^{2+} or Na^+ leads to an increase in fluorescence (by poising the $E1-E2$ equilibrium in the direction of $E1$), whereas K^+ (or Rb^+) and Pi in the presence of Mg^{2+}, produce a decrease in the signal, reflecting the stabilization of $E2$ states. ATP prevents eosin binding supporting the idea that eosin and the nucleotide bind to the same site [15, 16]. Note that in imidazole-HCl buffer at pH 7.4—about 7 mM imidazolium—a fraction of the enzyme is in the high-fluorescence state, indicating that eosin is already bound before the addition of Na^+ or Mg^{2+}, and suggesting that E-imidazolium is an $E1$ form.

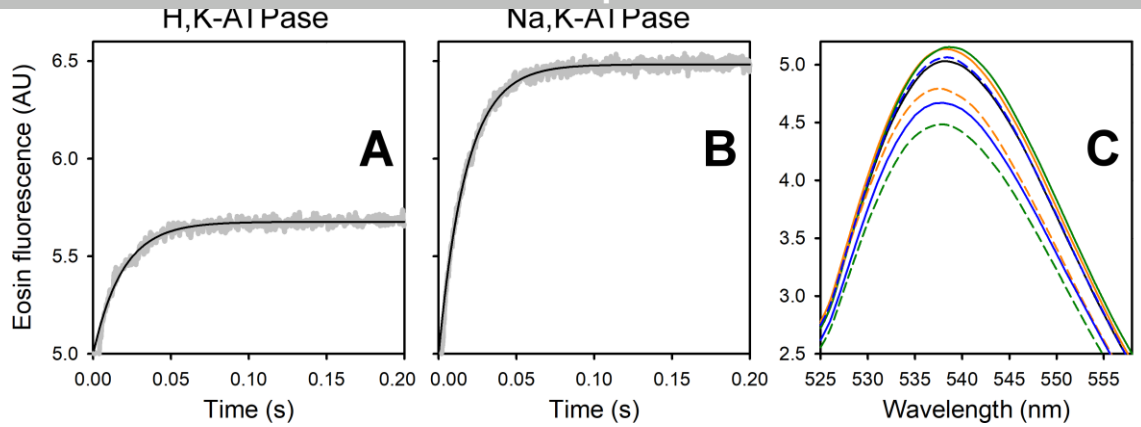

Figure 2. Eosin interaction. Time course of eosin binding to (A) H,K-ATPase and (B) Na,K-ATPase. Eosin fluorescence was measured upon mixing H,K-ATPase or Na,K-ATPase (50 µg of protein per ml, in the presence of 20 mM NaCl) with 0.4 µM eosin (all final concentrations). Continuous lines represent the best fitting of Eq. 1 to the data. (C) Fluorescence emission spectra for the H,K-ATPase (100 µg of protein per ml) incubated with 0.5 µM eosin alone (——) or with: 25 µM ATP (-----), 1 mM Mg^{2+} (——), 10 mM K^+ or Rb^+ (-----), 10 mM Na^+ (——), 3 mM Pi (-----), 3 mM Pi + 1 mM Mg^{2+} (——).

3.2. The interaction with Na^+ induces the E1 conformation.

Since Na^+ shifts the equilibrium to *E*1, we studied the time course of the *E*2→*E*1 conformational change induced by Na^+ in the H,K-ATPase and the Na,K-ATPase incubated with Rb^+ (Figure 3). In the case of the H,K-ATPase, fluorescence rise is almost complete in 0.7 seconds and the calculated k_{obs} value is $10.3 \pm 0.2\ s^{-1}$ (Figure 3A). In contrast, the k_{obs} value of the fluorescence change for the mammalian kidney Na,K-ATPase, $0.035 \pm 8\times10^{-5}\ s^{-1}$ (Figure 3B), is two orders of magnitude lower. As the *E*2K^+→*E*1 transition has been associated with the K^+ deocclusion process [19, 21], the high velocity found for the H,K-ATPase is in agreement with the low stability proposed for the state holding occluded K^+ [8]. Therefore, while K^+ deocclusion is accepted to be a limiting step in the mammalian kidney Na,K-ATPase cycle, this does not seem to be the case for the gastric H,K-ATPase.

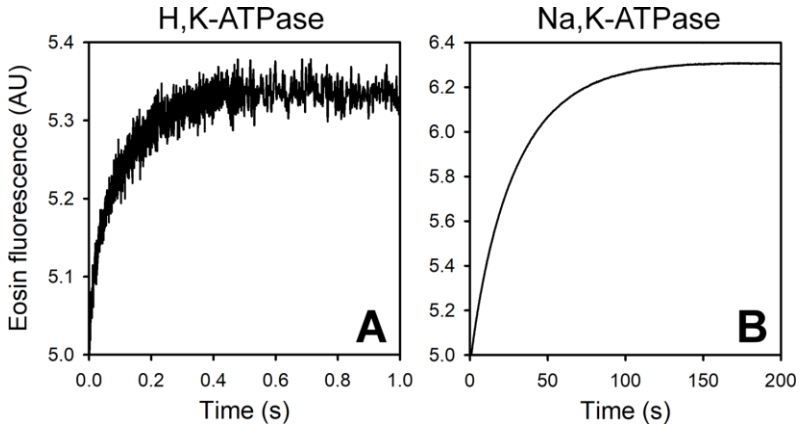

Figure 3. Time course of *E*2→*E*1 conformational transition. Eosin fluorescence was measured upon mixing H,K-ATPase or Na,K-ATPase (40 µg of protein per ml, 500 µM Rb^+ and eosin 0.4 µM) with 20 mM NaCl (all final concentrations).

Under equilibrium conditions, Na^+ increases the fluorescence for the H,K-ATPase incubated with Rb^+ (Figure 4A) with a $K_{0.5}$ of 4.3 ± 0.39 mM. A rise in fluorescence was also observed as a function of choline chloride (ChCl), used to test ionic strength. In the case of the Na,K-ATPase, it has been established that certain protonated buffers favor the *E*1 conformation [19, 22-24]; thus, it is also conceivable for choline, to increase eosin fluorescence in the H,K-ATPase preparation. As Na^+ has been proposed as a substitute for H^+ in the H,K-ATPase [4, 5], here we investigated its influence on the ATPase activity. We observed that Na^+ inhibits ATP hydrolysis with low affinity (Figure 4B), an effect that increases with pH. This agrees with previous data [3] and might be interpreted in terms of a competition between Na^+ and H^+, though this competition does not unequivocally imply that Na^+ is transported or even that it binds to the cation transport sites at all.

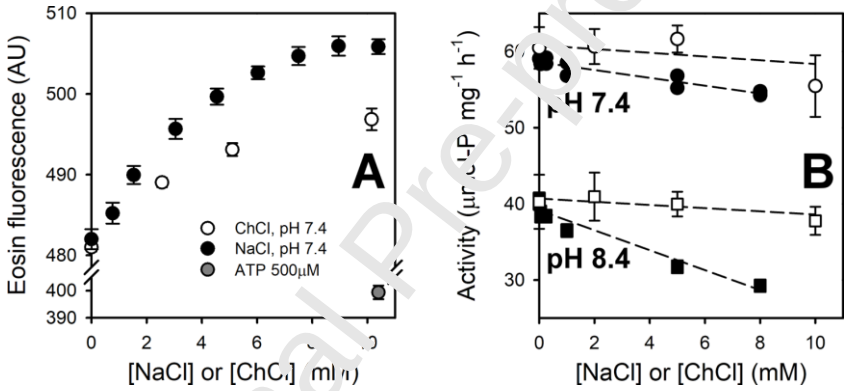

Figure 4. Effect of sodium on the H,K-ATPase. (A) Eosin fluorescence for the enzyme (40 µg of protein per ml) incubated with 0.4 µM eosin, 50 µM Rb^+ and varying concentrations of NaCl or choline chloride (ChCl). 500 µM ATP was added to control basal fluorescence. (B) ATPase activity was measured as a function of NaCl (full symbols) or ChCl (empty symbols) at pH 7.4 or 8.4. Values ± 1 S.E.

To evaluate the capacity of the H,K-ATPase to transport Na^+, we measured the ATPase-dependent uptake of this cation in the gastric vesicles. Vesicles were incubated with $^{22}Na^+$ for different lengths of time in the presence or absence of ATP; the pH was set to 8.4 to increase Na^+ affinity. Figure 5 shows the uptake of $^{22}Na^+$ in H,K-ATPase-rich vesicles. We observed a significant influx of Na^+ that is not related to the H,K-ATPase function, as already reported by Schackmann *et al.* [25]. However, the addition of ATP did not lead to an increase of $^{22}Na^+$ uptake under the same conditions used to measure ATPase activity (see Figure 4B). Therefore, under the experimental conditions used in our experiments, it was not possible to detect active transport of Na^+ by the H,K-ATPase.

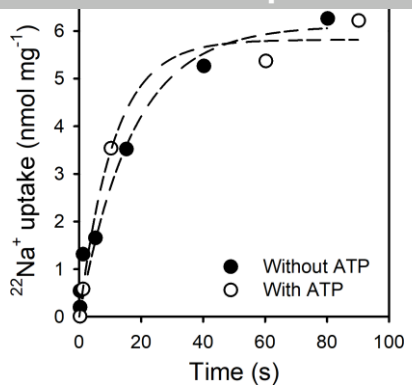

Figure 5. $^{22}Na^+$ uptake by H,K-ATPase-rich vesicles. Na^+ incorporation was measured upon mixing a suspension of H,K-ATPase vesicles (90 µg of protein per ml, incubated with 10 mM KCl, pH 8.4) with 6.0 mM $^{22}NaCl$, 3.0 mM $MgCl_2$, 10 mM KCl, in the presence or absence of 2.0 mM ATP (all final concentrations). Dashed lines were included to guide the eye.

The Albers-Post model for the Na,K-ATPase states that a Na^+-occluded state is an obligatory intermediate of the reaction cycle of an ATPase that actively transports Na^+ [25]. Recently, Ochiai *et al.* [26] showed that epigallocatechin-3-gallate (EGCg) inhibits Na,K-ATPase activity by stabilizing the *E*1P(Na) state, which holds occluded Na^+. In steady-state experiments (see Figure S2 in *Supplementary Material*), we observed that EGCg inhibits ATPase activity of both H,K-ATPase ($K_{0.5}$ = 10.82 ± 0.42 µM) and Na,K-ATPase ($K_{0.5}$ = 0.94 ± 0.14 µM). Accordingly, we tested the existence of a Na^+-occluded state in the H,K-ATPase using a filtration and washing technique [10, 18] in the presence of EGCg. The enzyme was incubated at pH 8.4 with different $^{22}Na^+$ concentrations. Figure 6 shows the levels of tightly bound $^{22}Na^+$ as a function of [Na^+] for active and thermally inactivated H,K-ATPase (the latter used as control of unspecific retention); we also performed some experiments with the Na,K-ATPase for comparison. Note the significant difference in Na^+ retention between inactivated and active Na,K-ATPase, with Na^+-occlusion values of 5-6 nmol per mg of protein, in addition to unspecific binding, at 12 mM Na^+ (Figure 6B). On the contrary, in the case of H,K-ATPase there is no difference at any of the tested Na^+ concentrations (Figure 6A). These results lead us to conclude that if an intermediate holding occluded Na^+ existed in the H,K-ATPase, it would be too transient to be detected.

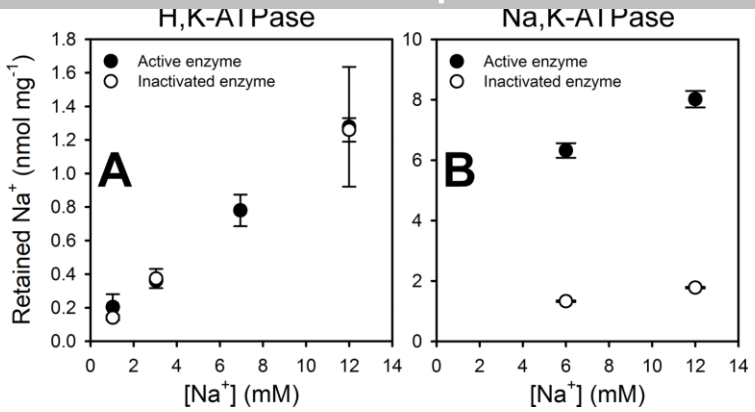

Figure 6. Equilibrium values of Na^+ tight binding. $^{22}Na^+$ retained on the filters for preparations of H,K-ATPase (pH 8.4) and Na,K-ATPase (pH 7.4). Enzymes (100 µg of protein per ml) were incubated with 1 mM EGCg, at varying concentrations of NaCl, and washed with ice-cold buffer containing 1 mM EGCg. Values ± 1 S.E.

3.3. Induction of E2: effect of K^+ and phosphate

The decrease of eosin fluorescence as a function of [K^+] and [Pi] (Figure 7) can be described by a hyperbolic function, $Y = Y_0\ K_{0.5}/([X] + K_{0.5})$, where Y is the normalized fluorescence value, Y_0 is the signal in the absence of ligands, and [X] is the ligand concentration. The best-fitting value of $K_{0.5}$ for *E*2K formation is 0.36 ± 0.03 mM (Figure 7A), a similar value to that reported for a FITC-labeled H,K-ATPase [27] and two orders higher than that reported for the Na,K-ATPase: 5-7 µM [28]. Since the high affinity for K^+ of the Na,K-ATPase has been related to the formation of a very stable *E*2K^+-occluded state [28], our results agree with the idea that the K^+ occluded state is less favored in the H,K-ATPase [4, 10]. It is worth mentioning that a much lower affinity for K^+ of the H,K-ATPase was found by Rabon *et al.* [4] ($K_{0.5}$ = 6 mM) in the presence of 150 mM ChCl.

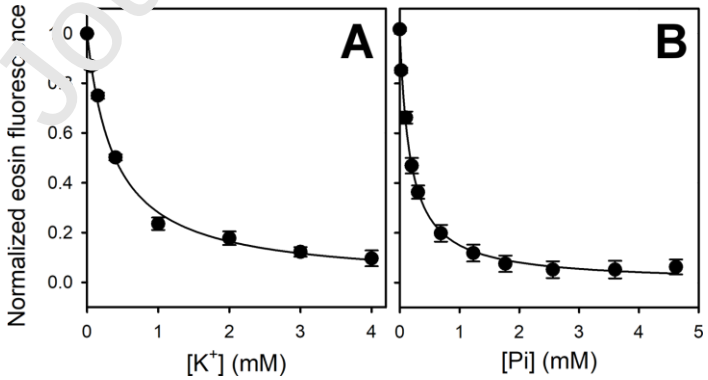

Figure 7. Effect of K^+ and phosphate on eosin fluorescence. The H,K-ATPase (40 µg of protein per ml) was incubated with 0.3 µM eosin, (A) as a function of [KCl] and (B) in the presence of 3.0 mM $MgCl_2$ as a function of [Pi]. Values ± 1 S.E. Continuous lines represent the best fitting of a rectangular hyperbola to the data.

In turn, Pi induces fluorescence decay (only in the presence of Mg^{2+}) with a $K_{0.5}$ of 0.17 ± 0.02 mM (Figure 7B), evidencing the formation of *E*2P. To investigate the mechanism of *E*2P formation it is necessary to use kinetic

determinations. Accordingly, we measured the time course of eosin fluorescence change at different Pi concentrations (Figure 8A). The decrease in fluorescence was fitted by an exponential function of time for each [Pi] tested (Eq. 2):

$$F(t) = \Delta F\, e^{-k_{obs}\, t} + F_{\infty} \qquad \text{Eq. 2}$$

where F_{∞} is the equilibrium fluorescence value, ΔF is the total fluorescence change and k_{obs} is the observed rate constant of the process. The calculated values of k_{obs} are plotted in Figure 8B as a function of Pi concentration. The fact that changes in fluorescence upon addition of Pi reflect the formation of *E*2P in the H,K-ATPase is supported by experiments of Pi phosphorylation by Van der Hijden *et al.* [29] who obtained a k_{obs} value of 0.06 - 0.09 s^{-1} for 0.06 mM Pi at 20 ºC, which is consistent with our results at 25 ºC.

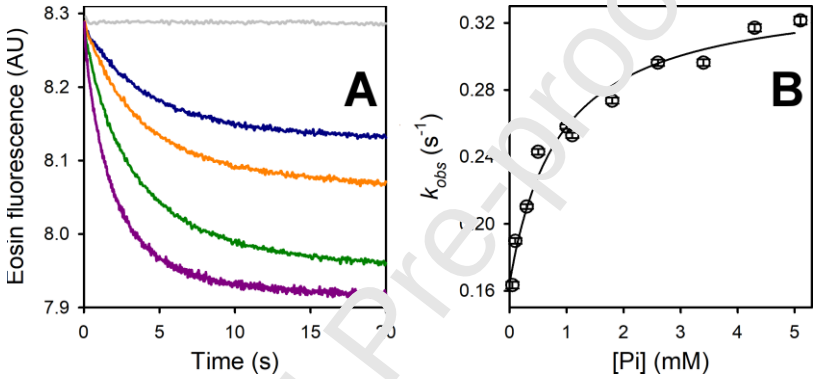

Figure 8. Kinetics of *E*2P formation in the H,K-ATPase. (A) Effect of Pi on the time course of fluorescence change. Eosin fluorescence was measured upon mixing H,K-ATPase (45 µg of protein per ml, 3.0 mM $MgCl_2$ and 0.4 µM eosin) with 0 (gray), 0.05 (blue), 0.10 (orange), 0.30 (green) or 5.00 mM (purple) Pi (all final concentrations). Experiments were performed in a stopped-flow spectrofluorometer. (B) Observed rate constant of eosin fluorescence change (values ± 1 S.E.) for varying concentrations of Pi were obtained by fitting Eq. 2 to the kinetic traces, some of which are shown in panel A. The continuous lines represent the plot of Eq. 3 for the following best-fitting values: k_r=0.34 ± 0.01 s^{-1}, k_{-r}=879 ± 244 s^{-1} and k_{off}=0.16 ± 0.01 s^{-1}—to avoid over-parameterization the value of k_{on} was fixed at 1000 mM^{-1} s^{-1}.

Figure 8B shows that k_{obs} increases with [Pi] in the H,K-ATPase. This behavior is opposite to that observed for the Na,K-ATPase—in which k_{obs} decreases with [Pi] [30, 31]—but similar to that obtained for the formation of *E*2P-like states as a function of the concentration of Pi-like compounds in the Ca-ATPase and Na,K-ATPase [32-34]. It is worth noting that the rise of k_{obs} as a function of Pi-like compounds in the Na,K-ATPase was explained by a mechanism in which the ligand is considered to bind in *rapid equilibrium* before the protein undergoes a conformational change, i.e., an *induced-fit* mechanism: $E \rightleftharpoons EL \rightleftharpoons E'L$ [33, 34]. This interpretation is based on enzyme inactivation results, which are unable to distinguish between *E*1 and *E*2 conformations. Since eosin fluorescence change is a direct consequence of the conformational transition, our analysis is more straightforward in terms of *E*1 and E2 and the reaction scheme should look as:

$$E1 \underset{k_{off}}{\overset{k_{on}[\mathrm{L}]}{\rightleftarrows}} E1\mathrm{L} \underset{k_{-r}}{\overset{k_r}{\rightleftarrows}} E2\mathrm{L} \qquad \textit{Induced-fit}$$

where k_{obs} increases hyperbolically with [L] according to $k_{obs} = k_r \frac{[\mathrm{L}]}{\mathrm{K_d}+[\mathrm{L}]} + k_{-r}$. Nevertheless, this scheme contradicts the Albers-Post model (Figure 1), in which Pi is released from *E*2P and—therefore—the reverse reaction is the binding of Pi to *E*2; this rather corresponds to a *conformational-selection* mechanism like:

$$E1 \underset{k_{-r}}{\overset{k_r}{\rightleftarrows}} E2 \underset{k_{off}}{\overset{k_{on}[\mathrm{L}]}{\rightleftarrows}} E2\mathrm{L} \qquad \textit{Conformational-selection}$$

where, assuming rapid-equilibrium binding, $k_{obs} = k_r + k_{-r} \frac{\mathrm{K_d}}{\mathrm{K_d}+[\mathrm{L}]}$. However, since this model determines that k_{obs} decreases with [L], it cannot be used to explain the behavior observed in Figure 8B. In this sense, Vogt and Di Cera [35] showed that if the rapid-equilibrium assumption is not invoked, in a *conformational-selection* mechanism k_{obs} is defined by Eq. 3 and can increase or decrease as a function of [L] depending on whether $k_{off} < k_r$ or $k_{off} > k_r$, respectively:

$$k_{obs} = \frac{k_r + k_{-r} + k_{off} + k_{on}[\mathrm{L}] - \sqrt{\left(k_{off} + k_{on}[\mathrm{L}] - k_r - k_{-r}\right)^2 + 4\, k_{-r}\, k_{on}[\mathrm{L}]}}{2} \qquad \text{Eq. 3}$$

Consequently, Eq. 3 was fitted to the data in Figure 8B (continuous line) for the values of the parameters listed in the figure legend. The good match with the results reveals that the increase in k_{obs} with ligand concentration, often associated with an *induced fit* [35], can also be explained by a *conformational selection*, as proposed by the Albers-Post model. For a more comprehensive description on this topic see the analysis of Figure S3 in *Supplementary Material*.

3.4. E2P dephosphorylation intermediates

During the transport cycle, K^+ binding leads to *E*2P dephosphorylation, which occurs through a series of consecutive intermediates: *E*2P ground state → *E*2P transition state → *E*2P product state (see the shaded area in Figure 1). To study the states that arise during this reaction, we analyzed the *E*2P species stabilized by phosphate-like compounds: ground (BeF_x), transition (vanadate), and product (MgF_x) states [33, 34, 36, 37]. Figure 9 shows the eosin fluorescence signal as a function of the concentrations of BeF_3^-, vanadate and F^- in the presence of Mg^{2+}. In panels A and B, results are described by hyperbolae as a function of ligand concentration; calculated $K_{0.5}$ values were 0.25 ± 0.02 and 0.09 ± 0.05 μM for BeF_3^- and vanadate, respectively. In panel C, we fitted a Hill equation and

obtained a $K_{0.5}$ of 1.55 ± 0.05 mM and a n_H of 3.1 ± 0.3, which agrees with the binding of more than one F^- to form the *E*2MgF_4 complex. It is worth noting that $K_{0.5}$ values are comparable with those obtained by measuring the residual ATPase activity as a function of the concentration of Pi analogs—*c.f.* Figure 2 in reference [36]. Hence, as in the case of Pi, the change in fluorescence would be reflecting the formation of the *E*2P-like states, and the appearance of these species is responsible for the loss of enzyme activity.

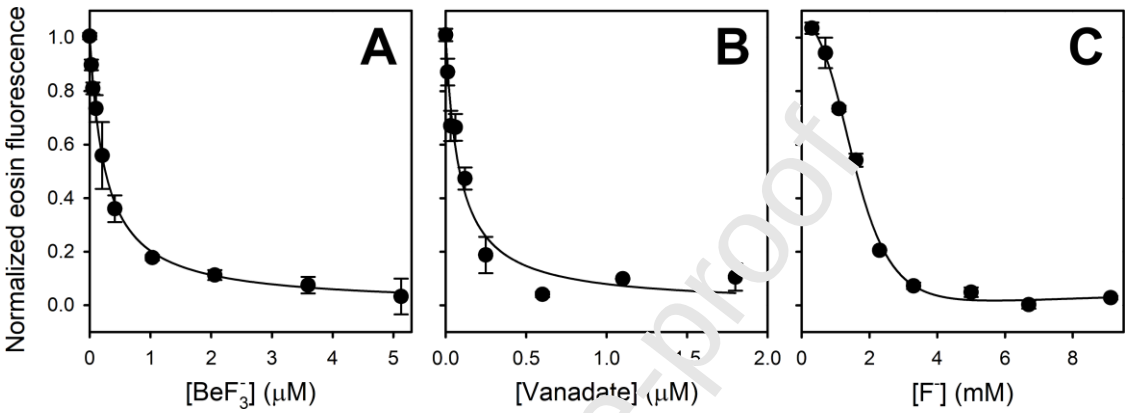

Figure 9. Effect of phosphate analogs on the equilibrium between *E*1 and *E*2P-like states. H,K-ATPase (40 μg of protein per ml) was incubated with 0.3 μM eosin. Fluorescence (values ± 1 S.E.) was measured in the presence of (A) 0.3 mM $MgCl_2$, 2.0 mM ImF and varying concentrations of $BeSO_4$, (B) 2.0 mM $MgCl_2$ and varying concentrations of vanadate, and (C) 2.0 mM $MgCl_2$ and varying concentrations of ImF. Continuous lines represent the best fitting for (panels A and B) $Y = Y_0 K_{0.5}/([X] + K_{0.5})$ and (panel C) $Y = Y_0 (K_{0.5})^{nH}/([X]^{nH} + (K_{0.5})^{nH})$.

The stability of these states was evaluated through the capacity of Na^+ to shift the equilibrium from the *E*2 to the *E*1 conformation. Figure 10 shows that, when the enzyme is incubated with Pi, the addition of 30 mM NaCl induces an increase of eosin fluorescence, reflecting the *E*2P→*E*1 conversion. Conversely, when the enzyme was incubated in the presence of BeF_x, the addition of Na^+ failed to trigger the shift to *E*1, whereas with vanadate or MgF_x the conversion was marginal. These results agree with those reported by Abe *et al.* [36], measuring the remaining ATPase activity, where the BeF_x-inhibited ATPase was found to be the most stable complex. The high stability of these complexes, evidenced in this experiment, explains the usefulness of phosphate analogs to obtain crystals [36, 38].

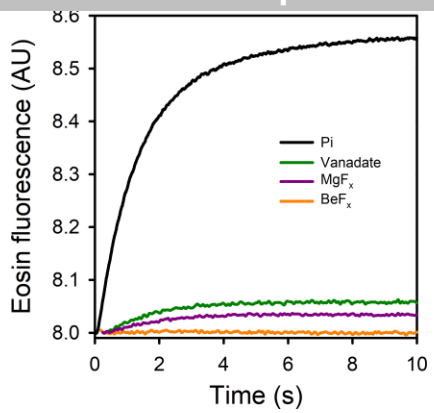

Figure 10. Effect of Na^+ on *E*2P and *E*2P-like states of the H,K-ATPase. A suspension of H,K-ATPase, incubated with eosin and either Pi or vanadate or MgF_x or BeF_x was mixed with a medium containing eosin and NaCl. Final concentrations were 60 µg of protein per ml, 0.4 µM eosin, 0.75 mM $MgCl_2$, 30 mM NaCl and either 200 µM Na_3VO_4 (vanadate), or 5 mM ImF and 2 mM $MgCl_2$ (MgF_x), or 1 mM ImF and 100 µM $BeSO_4$ (BeF_x), or 3 mM Pi.

We characterized the functionality of these *E*2P-like states in terms of their ability to occlude Rb^+ (as a congener of K^+). H,K-ATPase preparation was incubated with alamethicin and $^{86}Rb^+$, with or without BeF_x, vanadate or MgF_x. The amount of tightly bound Rb^+ (Rb_{occ}) shown in Table 1 was calculated subtracting the results obtained in experiments with inactivated enzyme from those obtained with active enzyme. As we have already shown [10], vanadate increases the amount of Rb_{occ} in the H,K-ATPase. Interestingly, the *E*2P-like state induced by MgF_x occludes an even higher amount of Rb^+, while the state induced by BeF_x shows almost no Rb^+ occlusion (the same as in the absence of added analogs). These results indicate the rearrangement of transmembrane helices associated with the change in phosphate geometry, where Rb^+ occlusion increases from the *E*2P-ground to the *E*2P-product state.

Table 1. Rb^+ occlusion (Rb_{occ}) in the H,K-ATPase.

Condition	Rb_{occ} (nmol Rb^+ per mg of protein)
No Pi analog	0.09 ± 0.01
BeF_x (0.75 mM $MgCl_2$, 100 µM $BeSO_4$ and 2 mM ImF)	0.10 ± 0.01
Vanadate (0.75 mM $MgCl_2$ and 0.2 mM Na_3VO_4)	0.22 ± 0.03
MgF_x (2 mM $MgCl_2$ and 8 mM NaF)	0.33 ± 0.06

H,K-ATPase (50 µg ml^{-1}) was incubated with 25 µg ml^{-1} alamethicin and 1 mM [^{86}Rb]RbCl.

4. Final Remarks

P-type ATPases present two basic conformational states, *E*1 and *E*2. Here we show that the measurement of eosin fluorescence time courses provides a reasonably precise method to study the kinetics of the *E*1−*E*2 conformational transition in the H,K-ATPase. We addressed the effect of Na^+, K^+ and Pi on functional and conformational features of the H,K-ATPase. The transport and occlusion of cations were studied using the radioactive isotopes $^{22}Na^+$ and $^{86}Rb^+$. The high similarity with the Na,K-ATPase led us to compare the properties of both proteins. The main findings of our work are:

- The assessment of Na^+ as a substitute for H^+ in the H,K-ATPase showed that Na^+: *(i)* induces a protein conformation with high nucleotide affinity—as H^+ would do according to the Albers-Post cycle (Figure 1)—and *(ii)* seems to compete with H^+ in ATPase activity experiments; however, it was not possible to isolate a state with occluded Na^+, neither to reveal an influx of Na^+ related to H,K-ATPase activity. These results suggest the inability of the enzyme to actively transport Na^+.

  The similarity between the ionic radius of Na^+ and H_3O^+, which was proposed to be the H^+-transported species in the H,K-ATPase [39, 40], supports the idea of Na^+ transport. However, it is interesting to consider the proton jumping mechanism, which proposes that amino acid residues, capable of H-bond formation, accept and donate H^+ [38, 41, 42], a model more difficult to reconcile with the ability to transport Na^+, though still compatible with Na^+ binding.

  In agreement with our data, competition between H^+ and Na^+ has been suggested from H^+ transport [3, 43], phosphorylation [44], and electrophysiological [45] measurements. Although the evidence supports the idea of Na^+ binding at the transport site in the H,K-ATPase, it cannot be ruled out the possibility of Na^+ ions screening charges on the surrounding membrane, which may electrostatically interact with the enzyme [24, 46].

- While K^+ deocclusion was proven to be a limiting step during the mammalian kidney Na,K-ATPase cycle [47, 48], our experiments show a high rate of the $E2K \rightarrow E1 + K^+$ reaction in the gastric H,K-ATPase even in the absence of ATP, which is known to accelerate this transition in the mammalian Na,K-ATPase [48, 49]. In the H,K-ATPase, a regulatory role of the nucleotide was proposed [50, 51] but also questioned [4, 8]; results in this work confirm the lack of a regulatory role of ATP. It is worth noting that a high rate for the *E*2→*E*1

transition was also reported in the shark Na,K-ATPase in which the limiting step under saturating concentrations of all ligands is proposed to be K^+ occlusion instead of K^+ deocclusion [52].

- We show for the first time the dependence of k_{obs} on [Pi] for *E*2P formation in the H,K-ATPase. Bearing in mind that *(i)* the values of k_{obs} increase as a function of [Pi] and that *(ii)* our measurements of eosin fluorescence are a direct consequence of the *E*1−*E*2 changes, an oversimplified evaluation of the results would lead to characterize the reaction as an *induced-fit* mechanism, i.e., that Pi binds to the *E*1 state. However, here we show that avoiding the rapid-equilibrium assumption, results can be explained by the binding of Pi to *E*2 (Eq. 3), which fits the Albers-Post model. It should be noted that the formation of *E*2P in the Na,K-ATPase, where k_{obs} decreases with Pi concentration [30, 31], can also be described by Eq. 3 given that $k_{off} > k_r$ [31, 35].
- Rb^+-occlusion results suggest that during the sequence of *E*2P hydrolysis, the transmembrane domain undergoes conformational changes from a marginally K^+-occluded (*E*2BeF_x) to an occluded (*E*2MgF_x) state, as outlined in Figure 11. This is in accordance with the fact that BeF_x induces an open conformation at the luminal side in the Ca-ATPase and H,K-ATPase [36, 37], and reduces the affinity for Rb^+ occlusion in the Na,K-ATPase [31].

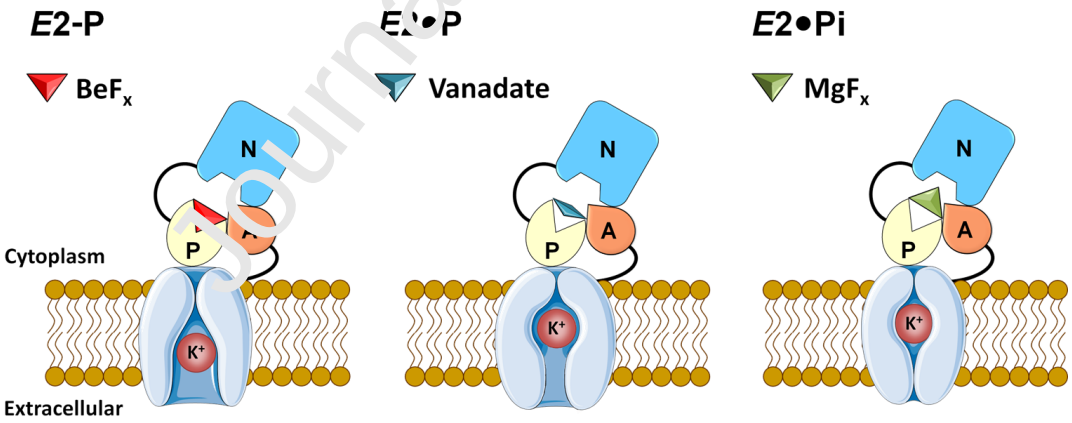

Figure 11. K^+ occlusion by different *E*2P-like states of the H,K-ATPase. Notice that Rb^+ occlusion is increasingly favored as the enzyme transitions from the *E*2P ground state (*E*2BeF_x) to the *E*2P product state (*E*2MgF_x).

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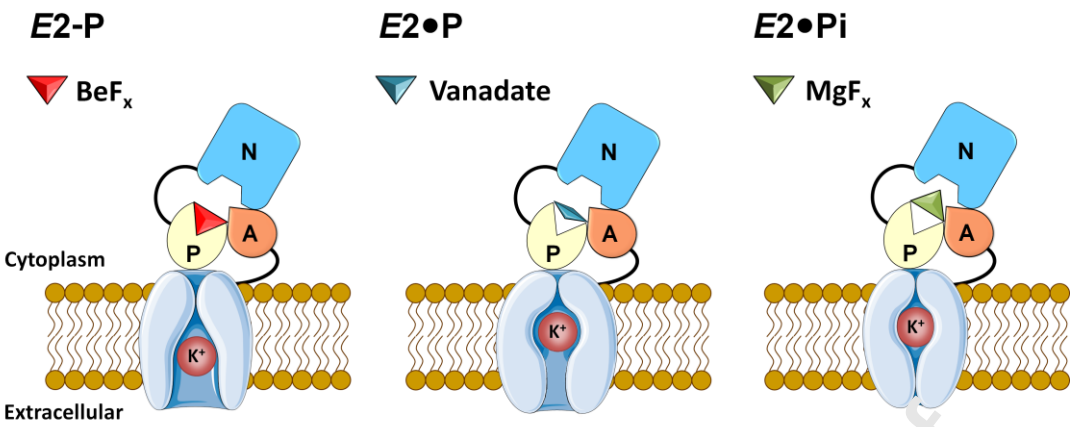
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- Eosin allows studying the kinetics of the *E*1−*E*2 transition in the H,K-ATPase
- Na^+ may substitute H^+ in some but not all types of interactions with the H,K-ATPase
- Neither occlusion nor active transport of Na^+ could be revealed in the H,K-ATPase
- Sharp contrast between the binding kinetics of Pi to H,K-ATPase and Na,K-ATPase
- There is a stepwise occlusion of K^+ during the *E*2P dephosphorylation sequence