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An unexpected effect of ATP on the ratio between activity and phosphoenzyme level of Na^+/K^+-ATPase in steady state ☆

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Abstract

According to the Albers–Post model the hydrolysis of ATP catalyzed by the Na^+/K^+-ATPase requires the sequential formation of at least two conformers of a phosphoenzyme (E_1P and E_2P), followed by the K^+-stimulated hydrolysis of E_2P. In this paper we show that this model is a particular case of a more general class of models in all of which the ratio between ATPase activity (v) and total phosphoenzyme level (EP) in steady state is determined solely by the rate constants of interconversion between phosphoconformers and of dephosphorylation. Since these are thought to be unaffected by ATP, the substrate curves for ATPase activity and EP should be identical in shape so that the ratio v/EP ought to be independent of the concentration of ATP. We tested this prediction by parallel measurements of v and EP as a function of [ATP] in the absence or presence of non-limiting concentrations of K^+, Rb^+ or NH_4^+. In the absence of K^+ or its congeners, both curves followed Michaelis–Menten kinetics, with almost identical K_m values (0.16 μM) so that v/EP remained independent of [ATP]. In the presence of either K^+, Rb^+ or NH_4^+, v and EP increased with [ATP] along the sum of two Michaelis–Menten equations. The biphasic response of v is well known but, to the best of our knowledge, our results are the first demonstration that the response of EP to [ATP] is also biphasic. Under these conditions, the ratio v/EP increased with [ATP] from 19.8 to 40.1 s^{-1} along a hyperbola that was half-maximal at 9.5 μM. To preserve the validity of the current model it seems necessary to assume that ATP acts on the $E_1P \rightleftharpoons E_2P$ transition and/or on the rate of hydrolysis of E_2P. The latter possibility was ruled out. We also found that to fit the Albers–Post model to our data, the rate constant for K^+ deocclussion from E_2 has to be about 10-times higher than that reported from measurements of partial reactions. The results indicate that the Albers–Post model quantitatively predicts the experimental behavior of the Na^+-ATPase activity but is unable to do this for the Na^+/K^+-ATPase activity, unless additional and yet unproved hypothesis are included.

Keywords: ATPase, Na^+/K^+- ; ATP hydrolysis; Phosphoenzyme; Albers–Post model

1. Introduction

The current reaction scheme for Na^+/K^+-ATPase [1–3] postulates that the hydrolysis of ATP proceeds through the sequential formation of at least one ADP-sensitive (E_1P) and one K^+-sensitive (E_2P) conformers of a phosphointermediate, followed by the hydrolysis of E_2P.

In media without K^+, ATP acts only at a high-affinity, catalytic site and Na^+-ATPase activity displays Michaelis–Menten kinetics as a function of the concentration of ATP with $K_m \cong 0.2$ μM see [4,5].

On the other hand, in the presence of K^+, ATP behaves not only as the substrate at the high-affinity site, but it also accelerates the release of occluded K^+, binding with low affinity ($K_m \cong 200$ μM) to E_2 [6]. As a consequence of this, the curve relating steady-state Na^+/K^+-ATPase activity to ATP concentration is biphasic and can be described by the sum of two Michaelis–Menten equations [7], irrespectively of the physical meaning of the parameters involved [4].

Although the Albers–Post model has solid experimental support, recent results are difficult to reconcile with this model, in particular when using rate constant values obtained from partial reactions to predict the behaviour of the overall activity. Experiments performed by Plesner et al. [8] and Rossi and Garrahan [5] show that the model fails to

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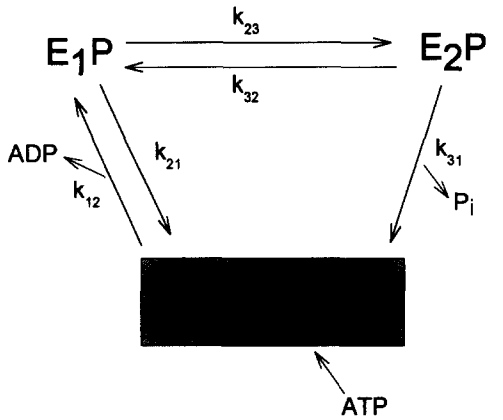


Fig. 1. A simplified reaction scheme for the hydrolysis of ATP by the Na^+/K^+-ATPase. Only the steps involved in the sequential formation of ADP-sensitive (E_1P) and K^+-sensitive (E_2P) conformers of a phosphoenzyme are shown. Dephosphorylation is considered irreversible because of the absence of P_i.

predict the behaviour of the overall activity. Difficulties were also found when trying to explain this activity through the kinetics of the phosphointermediates [8–10].

For these reasons, we decided to test properties that can be measured in steady-state conditions, such as ATPase activity and EP level. In the following we will show that the ratio between these two properties allows to investigate a small group of rate constants involved in the $E_1P \rightleftarrows E_2P$ interconversion and in the hydrolysis of E_2P.

The Albers–Post model is a particular case of the class of schemes shown in Fig. 1. In this scheme, the steady-state rate of ATP hydrolysis (v) in the absence of P_i will be

$$v = k_{31}E_2P = k_{23}E_1P - k_{32}E_2P \quad (1)$$

Since the total amount of phosphoenzyme (EP) is

$$EP = E_1P + E_2P \quad (2)$$

it follows that

$$EP = \frac{E_2P(k_{31} + k_{32} + k_{23})}{k_{23}} \quad (3)$$

Using Eqs. (1) and (3) we can obtain an explicit expression for the ratio between v and EP:

$$\frac{v}{EP} = \frac{k_{31}k_{23}}{k_{31} + k_{32} + k_{23}} \quad (4)$$

Eq. (4) shows one of the fundamental properties of the class of models in Fig. 1, i.e., that the ratio v/EP will be independent of the rates of phosphorylation and of transitions among the dephospho forms. It is important to stress that the constancy of the ratio v/EP is not a property of the simplified scheme of Fig. 1 but will also hold for reaction schemes including any amount of additional phosphoconformers, even if these were able to release P_i.

Eq. (4) also shows that if, as it is proposed by the Albers–Post model, the values of the rate constants that determine the ratio v/EP are not affected by ATP, then this ratio will be independent of the concentration of the nucleotide so that the functions relating ATPase activity and EP to the concentration of ATP will be identical in shape.

The prediction of the independence of v/EP with [ATP] was submitted to experimental test by parallel measurements in steady state of Na^+/K^+-ATPase activity and of EP as a function of [ATP], in media with and without non-limiting concentrations of either K^+, Rb^+ or NH_4^+.

Results showed that this prediction was satisfied for Na^+-ATPase activity, but not when K^+ or its congeners were present (Na^+/K^+-ATPase conditions). During these studies we also found that to fit the Albers–Post model to the experimental data, the rate constant for K^+ deocclusion from E_2 had to be about 10-times higher than the value reported from measurements of partial reactions.

2. Materials and methods

2.1. Reagents

[^{32}P]Orthophosphate was purchased from the Comisión Nacional de Energía Atómica of Argentina. ATP was labeled according to Glynn and Chappell [11], except that no orthophosphate was added. Enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were from Sigma. All other reagents were of analytical reagent grade.

2.2. Enzyme source

Na^+/K^+-ATPase from pig kidney outer medulla was kindly provided by Dr. J.G. Nørby (Institute of Biophysics, University of Århus, Denmark). It was prepared by the procedure of Jensen et al. [12]. The enzyme had a specific activity of 12 units/mg. Some experiments performed thereafter used an enzyme with a specific activity of 25 units/mg.

2.3. Protein determination

This was carried out by using the method of Lowry et al. [13].

2.4. Incubation media

Except where otherwise stated, the experiments were carried out at 25°C in media containing 150 mM NaCl, 0.2 mM EDTA, enough $MgCl_2$ to give a final concentration of 0.5 mM Mg^{2+}, 25 mM imidazole-HCl (pH 7.45 at 25°C) and 0 or 10 mM of either KCl, RbCl or NH_4Cl. The concentration of free magnesium was estimated from the total concentrations of ATP, EDTA and $MgCl_2$ using the equilibrium constants for the dissociation of Mg^{2+} from $Mg.ATP^{2-}$ and $Mg.EDTA^{2-}$ and the set of microscopic

dissociation constants of H^+ from $EDTAH_4$ and $ATPH_2^{2-}$ given by Dawson et al. [14].

2.5. ATPase activity

This was assayed as in Richards et al. [15], following the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$. Hydrolysis never exceeded 10% of the ATP present. Blanks had 1 mM ouabain.

2.6. Determination of EP

EP was measured as the amount of acid-stable ^{32}P incorporated to the enzyme from $[\gamma\text{-}^{32}P]ATP$. The phosphorylated and denatured protein (15–30 μg) was collected by filtration on Whatman GF/C or on Millipore filters (Type GS, 0.22 μm pore size) and washed three times with 10 ml of 7% (w/v) trichloroacetic acid and 32 mM H_3PO_4. Protein was quantitatively retained on the filters. These were placed in 5 ml of 0.4% (w/v) 2,5-diphenyloxazole, 0.02% (w/v) 1,3-bis-2-(5-phenyloxazole) in toluene for liquid scintillation counting. Blanks were measured in the same media without $MgCl_2$. They increased linearly with [ATP] and were at most 45% of the total EP level at the highest [ATP] tested.

2.7. Steady-state level of EP

The reaction was started by mixing 1 ml of $[\gamma\text{-}^{32}P]ATP$ with 1 ml of the enzyme suspension and quenched, after 1–3 s, by adding 3 ml of an ice-cold solution of 11% (w/v) trichloroacetic acid and 50 mM H_3PO_4.

2.8. Effect of ATP on the rate of dephosphorylation

Experiments were performed at 20 ± 1°C with a rapid mixing apparatus (Bio-Logic SFM-4, Claix, France) which allows to measure time-courses in the millisecond scale.

The enzyme suspension (20 μg protein/assay) was phosphorylated in 5 μM $[\gamma\text{-}^{32}P]ATP$ and 0.5 mM free Mg^{2+}. After 3 s, phosphorylation was stopped by addition of 10 mM of EDTA plus (final concentrations): (1) 1 mM Na^+; (2) 1 mM Na^+ and 200 μM ATP; (3) 1 mM K^+ or (4) 1 mM K^+ and 200 μM ATP. Chase lasted from 8 to 26 ms, after which the reactions were quenched with 20% (w/v) trichloroacetic acid and 91 mM H_3PO_4.

2.9. Statistical analysis

Theoretical equations were fitted to the experimental data by weighted non-linear regression using a modified Gauss–Newton routine. The standard deviation of both v and EP replicates were directly proportional to the average values of these magnitudes, typically about 6% of $\bar{v}$ and 10% of $\overline{EP}$. This allowed to calculate the weighting factors for the data of v or EP as $\bar{v}^{-2}$ or $\overline{EP}^{-2}$, respectively. Considering the size of the errors for v and EP and that they are normally distributed, a normal distribution of errors for v/EP was found to be a reasonably valid approximation at any concentration of ATP.

Table 1
Values of the parameters (±S.E.) that give best fit to v and EP as a function of [ATP] in media with K^+, Rb^+ or NH_4^+

Cation	$v = f([ATP])$		$EP = f([ATP])$		$(V_{m2}/EP_{m2})/(V_{m1}/EP_{m1})$
K^+	V_{m1}	0.12 ± 0.01	EP_{m1}	0.12 ± 0.02	
	K_{m1}^v	0.17 ± 0.03	K_{m1}^{EP}	0.41 ± 0.119	
	V_{m2}	3.32 ± 0.33	EP_{m2}	1.47 ± 0.36	
	K_{m2}^v	186 ± 35	K_{m2}^{EP}	264 ± 57	
					2.3 ± 0.6
NH_4^+	V_{m1}	0.09 ± 0.01	EP_{m1}	0.13 ± 0.02	
	K_{m1}^v	0.25 ± 0.14	K_{m1}^{EP}	0.17 ± 0.03	
	V_{m2}	4.04 ± 0.14	EP_{m2}	1.43 ± 0.17	
	K_{m2}^v	209 ± 13	K_{m2}^{EP}	194 ± 47	
					4.3 ± 0.94
Rb^+	V_{m1}	0.03 ± 0.01	EP_{m1}	0.03 ± 0.01	
	K_{m1}^v	0.10 ± 0.04	K_{m1}^{EP}	0.14 ± 0.07	
	V_{m2}	5.22 ± 1.43	EP_{m2}	1.69 ± 0.39	
	K_{m2}^v	433 ± 168	K_{m2}^{EP}	238 ± 97	
					3.6 ± 1.2

$$v = \frac{V_{m1}}{1 + \frac{K_{m1}^v}{[ATP]}} + \frac{V_{m2}}{1 + \frac{K_{m2}^v}{[ATP]}} \quad (5)$$

or

$$EP = \frac{EP_{m1}}{1 + \frac{K_{m1}^{EP}}{[ATP]}} + \frac{EP_{m2}}{1 + \frac{K_{m2}^{EP}}{[ATP]}} \quad (6)$$

V_m, EP_m and K_m values are in μmol mg^{-1} min^{-1}, nmol mg^{-1} and μM, respectively. Subscripts 1 and 2 denote the high- and low-affinity components, respectively.

The standard error of $f = (V_{m2}/EP_{m2})/(V_{m1}/EP_{m1})$ (last column in Table 1) was calculated as:

$$S.E.(f) = f\left(\frac{Var(V_{m1})}{V_{m1}^2} + \frac{Var(EP_{m1})}{EP_{m1}^2} + \frac{Var(V_{m2})}{V_{m2}^2} + \frac{Var(EP_{m2})}{EP_{m2}^2} - 2\frac{Cov(V_{m1},V_{m2})}{V_{m1}V_{m2}} - 2\frac{Cov(EP_{m1},EP_{m2})}{EP_{m1}EP_{m2}}\right)^{1/2}$$

where *Var* is the variance and *Cov* the covariance of the parameters between brackets. Only the covariances of the parameters obtained form the same set of data were calculated since the covariances of the rest are zero.

The sum of two Michaelis–Menten equations was fitted to the data of v and EP as function of [ATP] in the presence of K^+ or its congeners. We have shown elsewhere [4] that, provided that some constraints among rate coefficients are satisfied, this equation will be a mathematically valid description for mechanisms as distinct as: (i) two active sites for the same substrate; (ii) the substrate acting as such and as non-essential activator at either the same [6] or different sites, and (iii) random bisubstrate kinetics. Since the above mentioned constraints are fulfilled for the substrate curve of the Na^+/K^+-ATPase [4] we took advantage of the ability of the sum of two Michaelis–Menten equations to give simple interpretations of the data in terms of maximal effects and K_m values without loss in the rigor of the treatment.

3. Results

3.1. Steady-state Na^+-ATPase activity and total phosphoenzyme level in the absence of K^+ or its congeners

Na^+-ATPase activity was a Michaelis–Menten function of the concentration of ATP with $V_m = 0.14 \pm 0.002$ μmol P_i/(mg min) and $K_m^v = 0.16 \pm 0.01$ μM. EP followed the same kind of function of [ATP], reaching a maximum of 2.37 ± 0.04 nmol/mg with a K_m^{EP}, of 0.16 ± 0.07 μM. The almost identical values of K_m determined that the ratio v/EP remained independent of [ATP]. Its value was 1.03 ± 0.06 s^{-1} (Fig. 2).

3.2. Steady-state level of Na^+/K^+-ATPase activity and total phosphoenzyme level as a function of [ATP]

The effect of ATP in concentrations ranging from 0.05 to 500 μM on v and EP was measured in media containing 10 mM of either K^+, Rb^+ or NH_4^+. For each K^+ congener, EP and v were biphasic functions of [ATP] that could be fitted by the sum of two Michaelis–Menten equations (Eqs. (5) and (6) in Table 1). From the best-fitting parameters given in Table 1 it can be seen that the main difference among the K^+ congeners was that V_{m1} and EP_{m1} in media with Rb^+ were lower than in media with NH_4^+ or K^+ while the values of EP_{m2}, V_{m2}, K_{m2}^v and K_{m2}^{EP} (i.e., the 'low-affinity components') for K^+, NH_4^+ and Rb^+ were not significantly different from each other.

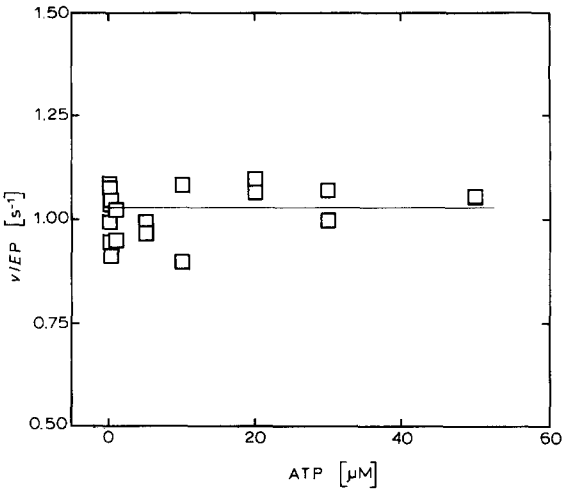


Fig. 2. The ratio v/EP as a function of the concentration of ATP during Na^+-ATPase activity. The data shown correspond to two independent determinations of v and EP in each of which duplicates were run. The plotted data are calculated from the average values of these measurements.

Using Eqs. (5) and (6) in Table 1, it can be proved that v/EP will be independent of [ATP] only if $K_{m1}^v = K_{m1}^{EP}$, $K_{m2}^v = K_{m2}^{EP}$ and $V_{m1}/EP_{m1} = V_{m2}/EP_{m2}$. Results in Table 1 show that, within the experimental error, the equalities between the corresponding K_m values are satisfied for each of the three cations tested, but that V_{m2}/EP_{m2} is from 2 to 4 times higher than V_{m1}/EP_{m1}. Therefore v/EP will change with [ATP]. This is illustrated in Fig. 3, where the mean value of v/EP for each of the three cations is plotted against the concentration of the nucleotide. It can be seen that the ratio increases about 2-fold as [ATP] goes from 0.1 to 100 μM and remains approximately constant at higher concentrations of the nucleotide. Results also show that this response does not vary significantly with the cations. For this reason the whole set of data was fitted together, averaging the values for the three cations (see inset to Fig. 3). A rectangular hyperbola (continuous line in the figure) was fitted to the values with v/EP going from 19.8 ± 1.1 to 40.1 ± 1.2 s^{-1} as [ATP] increases from zero to infinity, and with $K_{0.5} = 9.52 \pm 6.7$ μM.

The above-mentioned results were confirmed measuring v and EP in the presence of 0 or 10 mM K^+, at 10 or 100 μM ATP, using a different enzyme preparation with higher specific activity (25 units/mg). Results showed that, when going from 10 to 100 μM ATP, v/EP remained constant in the absence of K^+ (0.79 ± 0.02 s^{-1}), while it increased 1.75-fold (from 32 ± 3 s^{-1} to 56 ± 13 s^{-1}) in the pres-

ence of 10 mM K^+, a finding that agrees with the experiments in Figs. 2 and 3.

Since EP was measured during a few seconds and v during 10 min or more, the variation of v/EP could have been caused by time dependent inactivation of the ATPase in media with K^+ and low [ATP] during the measurement of v. To test this possibility, a Na^+/K^+-ATPase preparation was preincubated in media with 0.5 mM Mg^{2+}, 150 mM NaCl, 25 mM imidazole-HCl (pH 7.45 at 25°C) complemented with either: (i) 0 K^+ + 0 ATP; (ii) 0 K^+ + 5 μM ATP; (iii) 10 mM K^+ or (iv) 10 mM K^+ + 5 μM ATP. After 10 min, enough ATP, KCl and $MgCl_2$ were added to all the media to give final concentrations of 3, 10 and 3 mM, respectively. Activity was measured and found to be the same irrespective of the composition of the preincubation media, indicating that no selective inactivation of Na^+/K^+-ATPase had occurred in the presence of K^+ and low ATP concentrations. This was confirmed by an independent experiment in which activity in media with 10 mM K^+ and 5 μM ATP (as in condition (iv)) was found to remain constant for at least 25 min.

An artificial change in v/EP could also have been generated by over or underestimation of the blanks for v and/or EP, depending on the concentration of ATP. However, this could be ruled out since both the blanks for EP and for v were linear functions of the concentration of ATP.

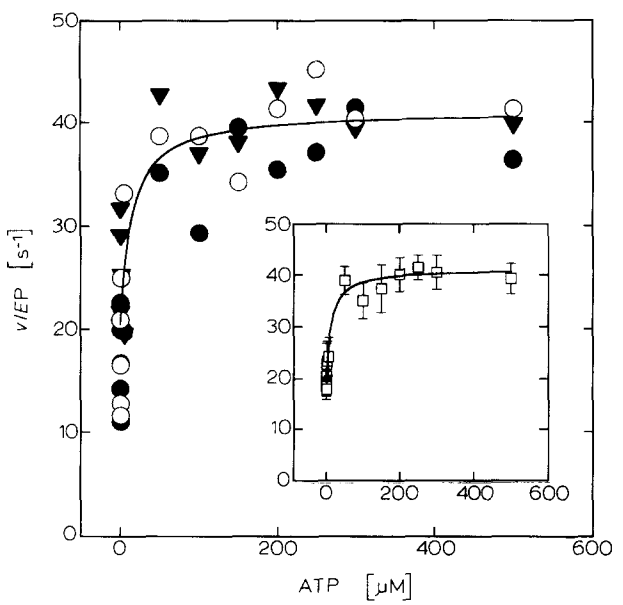


Fig. 3. Dependence of the ratio v/EP with the concentration of ATP. Each point is the average value of the ratio for K^+ (▼), NH_4^+ (○) or Rb^+ (●), taken from two independent experiments run in duplicate for each cation. The continuous line is the solution of

$$\frac{v}{EP} = \frac{(v/EP)_0 K_{0.5} + (v/EP)_m [ATP]}{K_{0.5} + [ATP]}$$

for the values given in the text. Inset: The values are means (±S.D.) obtained by averaging v/EP over all the cations used.

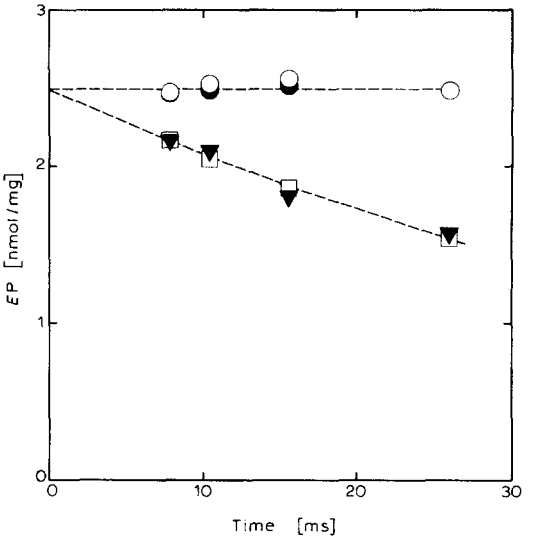


Fig. 4. Lack of effect of ATP on the time-course of dephosphorylation. EP was formed in the presence of 150 mM NaCl, 0.2 mM EDTA, 0.5 mM Mg^{2+}, 25 mM imidazole-HCl (pH 7.45 at 25°C) and 5 μM [γ-^{32}P]ATP. After 3 s, EP was chased (time = 0) by addition of 10 mM of EDTA plus (final concentrations): (i) 1 mM Na^+ (○); (ii) 1 mM Na^+ and 200 μM ATP (●); (iii) 1 mM K^+ (▼) or (iv) 1 mM K^+ and 200 μM ATP (□). Reactions were quenched after 8, 10, 16 or 26 ms with 20% (w/v) trichloroacetic acid and 92 mM H_3PO_4. The horizontal line is the average level of EP in the absence of K^+. In media with K^+, the curve fitted to the points represents EP = 2.5 $e^{-(18\,s^{-1})t}$ nmol/mg.

In view of these results, a kinetic explanation for the variation in v/EP seemed necessary. For the model in Fig. 1 this means that there should be effects of ATP on the rate constants in Eq. (4). Among these, only k_{31} can be estimated unambiguously and directly measuring the rate of K^+-dependent dephosphorylation of EP.

In the experiments shown in Fig. 4, dephosphorylation was initiated by addition of an excess of EDTA to media with and without 1 mM K^+ and 200 μM ATP. K^+ concentration had to be lowered because at 20°C dephosphorylation in media with 10 mM K^+ was too fast for the time resolution of the rapid-mixing apparatus. In the absence of K^+, no significant decay of EP was detectable after 26 ms, regardless of the presence or absence of ATP. In the presence of K^+, the apparent dephosphorylation rate constant was 18 s^{-1} and was not modified by ATP. In a different experiment (not shown), phosphorylation was not stopped by excess EDTA and dephosphorylation was measured following the decay of EP to a new steady state after the addition of 1 mM K^+. Neither in this case did we find an effect of ATP on the rate of dephosphorylation. Our dephosphorylation experiments at 20°C confirmed similar experiments performed at 0°C by Klodos and Nørby [16].

4. Discussion

We have shown in this paper that for kinetic schemes like that in Fig. 1, v/EP will depend exclusively on the

rate constants for the conformational changes and the hydrolysis of the phosphoenzyme. Hence if, as proposed by the current versions of the Albers–Post model, these were not affected by ATP, v/EP would be independent of the concentration of the nucleotide.

During Na^+-ATPase activity, our results fitted this prediction. In media with K^+ or its congeners, v and EP varied with the concentration of ATP along the sum of two Michaelis–Menten equations. Since its detection by Neufeld and Levy in 1969 [7], this response is well known for the case of v but, to the best of our knowledge, ours is the first demonstration that the response of EP to [ATP] is also biphasic. As shown in Section 1, this is predicted by the Albers–Post scheme. However, to keep v/EP independent of [ATP], it is also required that the functions relating v and EP to [ATP] be identical in shape. This was not satisfied by our results which showed that v/EP increased 2-fold as [ATP] went from zero to infinity.

This effect of ATP could not be accounted for by enzyme inactivation or by an artifact in the distribution of blanks. Therefore, to preserve the model in Fig. 1 it seems necessary to include an effect of ATP on the rates of hydrolysis of E_2P and/or of the $E_1P \rightleftharpoons E_2P$ transition (see Eq. 4).

ATP did not change the rate of dephosphorylation, so that effects of the nucleotide on this reaction can be dismissed. v/EP would also increase if ATP reduced the rate of the $E_2P \rightarrow E_1P$ transition (k_{32} in Fig. 1). However, as this transition is supposed to occur only on the fraction of E_2P that is free of K^+, such an effect can be disregarded because saturating concentrations of K^+ were used. Hence, an effect of ATP on k_{23} would be the most likely explanation for our findings. To see if this could predict quantitatively our results, we solved the steady-state equations for v and EP as a function of [ATP] for the model proposed by Moczydlowski and Fortes [6] which uses a single alternating site to explain the biphasic behaviour observed in the ATPase activity in the presence of K^+ (Fig. 5). To include an effect of ATP on k_{23} we modified the model in Fig. 5 making k_3 (k_{23} in Fig. 1) the following function of [ATP]:

$$k_{3app} = \frac{(k_{30}K_{ATP} + k_{3m}[ATP])}{K_{ATP} + [ATP]} \quad (7)$$

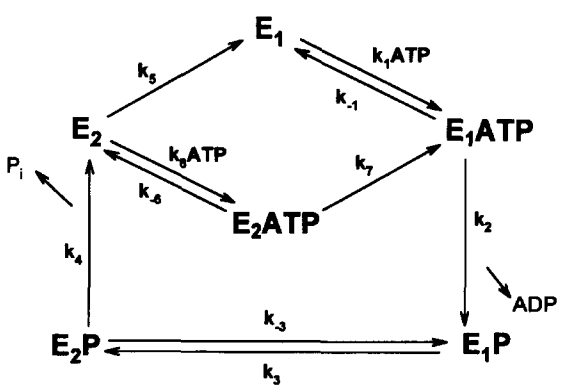
Eq. (7) would hold if the $E_1P \rightarrow E_2P$ transition occurred through two parallel pathways: an ATP-independent one, with rate constant k_{30}, and an ATP-dependent one with rate constant k_{3m}. As [ATP] goes from 0 to infinity k_{3app} increases from k_{30} to k_{3m} becoming equal to $(k_{30} + k_{3m})/2$ when $[ATP] = K_{ATP}$. The hyperbolic nature of Eq. (7) agrees with the shape of the effect of [ATP] on v/EP found experimentally.

To obtain values for the constants in Eq. (7) that are compatible with the experimental results we started from the fact that, for the model in Fig. 5 at non-limiting concentrations of K^+ or its congeners:

$$\frac{v}{EP} = \frac{k_3 k_4}{k_3 + k_4} \quad (8)$$

Eq. (8) is similar to Eq. (4), except that, for the reasons already given, k_{-3} is absent. Replacing Eq. (7) into Eq. (8), we obtain

$$\frac{v}{EP} = \frac{(v/EP)_0 K_{0.5} + (v/EP)_m[ATP]}{K_{0.5} + [ATP]} \quad (9)$$



	Na^+-ATPase	Na^+/K^+-ATPase
k_1	10 $s^{-1}\mu M^{-1}$	10 $s^{-1}\mu M^{-1}$
k_{-1}	2 s^{-1}	2 s^{-1}
k_2	180 s^{-1}	180 s^{-1}
k_3	48 s^{-1}	48 s^{-1}
k_{-3}	14.5 s^{-1}	14.5 s^{-1}
k_4	2.5 s^{-1}	233 s^{-1}
k_5	60 s^{-1}	0.26 s^{-1}
k_6	-------	1.6 $s^{-1}\mu M^{-1}$
k_{-6}	-------	833 s^{-1}
k_7	-------	54 s^{-1}

Fig. 5. The Albers-Post scheme for Na^+/K^+-ATPase activity. Phosphorylation and dephosphorylation were considered irreversible because in our experimental conditions [ADP] and [P_i] were close to 0. The transitions $E_2 \rightarrow E_1$ and $E_2ATP \rightarrow E_1ATP$ were taken as irreversible since in all the experiments the concentration of Na^+ was sufficiently high as to shift the equilibrium between conformers towards the E_1 forms (Glynn and Richards [19]). The Na^+-ATPase cycle was considered to occur exclusively through the $E_2 \rightarrow E_1 \rightleftarrows E_1ATP$ pathway. The inset shows the values of the rate constants used for the calculations in the text. k_3 was taken as equal to k_{3m} (see comments to Eqs. (10) to (12)), k_{-3} was calculated assuming that $k_{-3}/k_{3m} \cong 0.3$ as proposed by Mårdh and Lindahl [24]. The values of the other constants were taken from the literature (for references see Rossi and Garrahan [5]). Higher values for k_1 have been proposed by Campos and Beaugé [23].

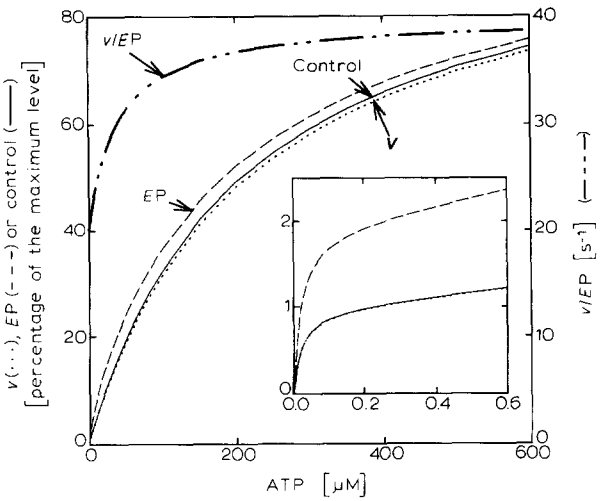


Fig. 6. Steady-state solutions of the model in Fig. 5 for the effects of [ATP] on v (...), EP (- - -) and v/EP (– – –), when k_3 is a hyperbolic function of [ATP]. For details see the main text. A control curve (—) corresponding to the Albers–Post model was calculated fixing k_3 at 48 s^{-1}, independently of [ATP]. Except in the case of v/EP, the data are expressed as percentage of its maximum value. The inset illustrates the initial part of the curves for v and EP.

where

$$(v/EP)_0 = \frac{k_4 k_{30}}{k_4 + k_{30}} \tag{10}$$

$$(v/EP)_m = \frac{k_4 k_{3m}}{k_4 + k_{3m}} \tag{11}$$

$$K_{0.5} = K_{ATP}\frac{k_4 + k_{30}}{k_4 + k_{3m}} \tag{12}$$

Fixing k_4 at 233 s^{-1} and using the values for $(v/EP)_0$ $(v/EP)_m$ and $K_{0.5}$ that gave best fit to the data in Fig. 3, we obtained $k_{30} = 22\ s^{-1}$; $k_{3m} = 48\ s^{-1}$ and $K_{ATP} = 40.75$ μM. With these values and those in Fig. 5, the substrate curves for v, EP and v/EP of Fig. 6 were generated. Except for v/EP, data are expressed as percentage of their maximum values. For comparative purposes we included a control curve corresponding to the standard Albers–Post model with $k_3 = 48\ s^{-1}$, independently of [ATP]. As already explained, this curve will be valid for both EP and v. It can be seen that as a consequence of the effect of ATP on k_{3app} the values of EP at low [ATP] were higher than, and those of v practically identical to those of the control curve. The ratio between these two curves (v/EP curve in Fig. 6) correctly reproduced the hyperbolic response of the experimental data. Hence an effect of ATP on k_{3app} as that shown in Eq. (7) predicts the observed response of v/EP to [ATP].

Although K^+ and its congeners are able to stimulate to the same extent, albeit with different affinity, the rate of dephosphorylation, it has been proposed that they have different effects on the $E_2 \rightarrow E_1$ transition [2]. Our observation that the functions relating v/EP to [ATP] were the same for K^+, Rb^+ and NH_4^+ is consistent with the class of models in Fig. 1, since in them v/EP is not affected by transitions among the dephospho forms (Eq. 4).

A central postulate of the Albers–Post model is that the reactions of phosphorylation and of interconversion between phospho forms are shared by the Na^+- and Na^+/K^+-ATPase activities. This raises the question of why the ratio v/EP remained constant during Na^+-ATPase activity if k_{3app} increased with [ATP]. To explain this without introducing additional modifications, it is necessary to suppose that, under our experimental conditions, most of EP is E_2P [17] so that it will be hardly affected by an increase in k_{3app}. This would mean that the actual ratio $(k_{-3} + k_4)/k_3$ is much lower than that predicted by the values in Fig. 5.

The control curve in Fig. 6 can be described by the sum of two Michaelis–Menten equations showing that theoretical and experimental results can be fitted by the same type of function. However the theoretical values of $K_{m1}^v = K_{m1}^{EP} = 0.02$ μM and $V_{m1} = EP_{m1} = 0.97\%$ are about ten times smaller than the experimental ones (Table 1). This discrepancy has been already reported by us [5]. If the model in Fig. 5 held, to explain this the value of at least one of the rate constants used to calculate the theoretical curves has to be different to its actual value. To detect which constant is this, we derived the expressions for K_{m1}^v, K_{m1}^{EP}, V_{m1} for the scheme in Fig. 5. These are

$$K_{m1}^v = K_{m1}^{EP} = \frac{k_5}{k_1}\left[\frac{1 + \frac{k_{-1}}{k_2}}{1 + \frac{k_5}{k_2} + \frac{k_5}{k_3} + \frac{k_5}{k_4}\left(1 + \frac{k_{-3}}{k_3}\right)}\right] \tag{13}$$

and

$$V_{m1} = k_5\left[1 + \frac{k_5}{k_2} + \frac{k_5}{k_3} + \frac{k_5}{k_4}\left(1 + \frac{k_{-3}}{k_3}\right)\right]^{-1} E_T \tag{14}$$

where E_T is the total enzyme concentration.

Using the values in Fig. 5 the terms between brackets become 1.003 and 1.01 for Eqs. (13) and (14), respectively. Therefore little error is committed if we consider

$$K_{m1}^v = K_{m1}^{EP} \cong k_5/k_1 \tag{15}$$

$$V_{m1} \cong k_5 E_T \tag{16}$$

Eqs. (15) and (16) pinpoint to the value of k_5 as the responsible for the discrepancy between the measured and the theoretical values of K_{m1}^v, K_{m1}^{EP} and V_{m1}. This is true both for the Albers–Post model and for the modified model that accounts for the effect of ATP on v/EP, since if k_3 is taken as equal to k_{30} (22 s^{-1}) the terms between brackets will become 0.996 and 1.02 for Eqs. (13) and (14), respectively.

The value of k_5 for the Na^+/K^+-ATPase that will fit the experimental data to the model in Fig. 5 can be

Table 2
Rate constants for dephosphorylation in the Na^+-ATPase cycle (k_4) or for the spontaneous deocclussion of either K^+, Rb^+ or NH_4^+ (k_5 in the Na^+/K^+ (or congener)-ATPase cycle)

ATPase	Rate constant estimated from v_i (s^{-1})	Rate constant estimated from EP (s^{-1})
Na^+/K^+	1.7 ± 0.3	4.1 ± 1.2
Na^+/NH_4^+	2.5 ± 1.4	1.7 ± 0.3
Na^+/Rb^+	1.0 ± 0.4	1.4 ± 0.7
Na^+	1.6 ± 0.1	1.6 ± 0.7

Values ($\pm$S.E.) were estimated by multiplying the K_m values by 10 $\mu M^{-1}s^{-1}$.

estimated multiplying the K_m values of Table 1 by the reported value of k_1 (see Eq. 15). The results of this operation are given in Table 2. It can be seen that, regardless of the K^+ congener used, to fit our results k_5 should be about 10 times higher than that given in Fig. 5. Notice that even with $k_5 = 4.1\ s^{-1}$, the Eqs. (15) and (16) would still hold, since in this case the terms between brackets would be 0.89 and 1.13, respectively. An additional evidence for the need of a higher value for k_5 is that the experimentally measured values of K_{m1}^{v}, K_{m1}^{EP} and V_{m1} of the Na^+/K^+-ATPase and K_m^v, K_m^{EP} and V_m of the Na^+-ATPase are similar (Table 1). We have previously shown [4] that, using the scheme in Fig. 5 for the Na^+-ATPase, $K_m^v \cong k_4/k_1$ and $V_m \cong k_4 E_T$. Comparing these expressions with Eqs. (15) and (16) it is clear that the value of k_5 for the Na^+/K^+-ATPase cannot be very different from that of k_4 for the Na^+-ATPase. We calculated k_4 following the same procedure used to estimate k_5. Comparison of k_4 in Table 2 with that in Fig. 5 shows that, in contrast with the case of k_5, there is a good agreement between the reported and the calculated value of k_4.

The value of k_5 in Fig. 5 was obtained measuring the rate of change in intrinsic fluorescence [18] or the rate of $^{86}Rb^+$ deocclusion [19,20] under pre-steady state conditions. Our values of k_5 correspond to experiments in steady state. Hence the inadequacy of the value of k_5 in Fig. 5 to explain our results may reflect the difference between the steady-state and pre-steady-state values of rate constants postulated by Peluffo et al. [21] and Rossi and Nørby [22].

In conclusion, results in this paper indicate that the Na^+-ATPase satisfied the tested predictions of the Albers–Post model but the Na^+/K^+-ATPase did not. It may be possible to preserve this model by including additional features that are only apparent during Na^+/K^+-ATPase activity. Since no evidence for such additional features is yet available, it remains an open question whether they are the cause of the discordance between theory and experiment or whether these denote an intrinsic flaw of the Albers–Post model.

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References

[1] Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727–756.
[2] Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.
[3] Glynn, I.M. (1985) in The enzymes of Biological Membranes, 2nd Edn. (Martonosi, A., ed.), pp. 33–114, Plenum Press, New York.
[4] Rossi, R.C. and Garrahan, P.J. (1985) in The Sodium Pump (Glynn, I.M. and Ellory, J.C., eds.), pp. 443–455, Cambridge University Press, London.
[5] Rossi, R.C. and Garrahan, P.J. (1989) Biochim. Biophys. Acta 981, 85–94.
[6] Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2357–2366.
[7] Neufeld, A.H. and Levy, H.M. (1969) J. Biol. Chem. 244, 6493–6497.
[8] Plesner, I.W., Plesner, L., Nørby, J.G. and Klodos, I. (1981) Biochim. Biophys. Acta 643, 483–494.
[9] Klodos, I., Nørby, J.G. and Plesner, I.W. (1987) Biochim. Biophys. Acta 643, 463–482.
[10] Nørby, J.G, Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725–759.
[11] Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147–149.
[12] Jensen, J., Nørby, J.G. and Ottolenghi, P. (1984) J. Physiol. 346, 219–241.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969) Data for Biochemical Research, pp. 426–427, p. 433, Oxford University Press, London.
[15] Richards, D.E., Rega, A.F. and Garrahan, P.J. (1978) Biochim. Biophys. Acta 511, 194–201.
[16] Klodos, I. and Nørby, J.G. (1988) in The Na^+,K^+-Pump. (Skou, J.C., Nørby, J.G., Maunsbach, A.B. and Esmann M., eds.), Part A, pp. 321–326, Allan R. Liss, New York.
[17] Klodos, I. and Nørby, J.G. (1987) Biochim. Biophys. Acta 897, 302–314.
[18] Karlish, S.J.D. and Yates, D.W. (1978) Biochim. Biophys. Acta 527, 115–130.
[19] Glynn, I.M. and Richards, D.E. (1982) J. Physiol. 330, 17–43.
[20] Forbush, B., III (1987) J. Biol. Chem. 262, 11104–1115.
[21] Peluffo, R.D., Garrahan, P.J. and Rega, A.F. (1992) J. Biol. Chem. 267, 6596–6601
[22] Rossi, R.C. and Nørby, J.G. (1993) J. Biol. Chem. 268, 12579–12590.
[23] Campos, M. and Beaugé, L.A. (1992) Biochim. Biophys. Acta 1105, 51–60
[24] Mårdh, S. and Lindahl, S (1977) J. Biol. Chem. 252, 8058–8061.