Biochimica et Biophysica Acta, 643 (1981) 483—494
Elsevier/North-Holland Biomedical Press

BBA 79223

THE STEADY-STATE KINETIC MECHANISM OF ATP HYDROLYSIS CATALYZED BY MEMBRANE-BOUND ($Na^+ + K^+$)-ATPase FROM OX BRAIN

III. A MINIMAL MODEL

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(Received August 6th, 1980)
(Revised manuscript received November 25th, 1980)

Key words: ($Na^+ + K^+$)-ATPase, Steady-state kinetics; K^+ inhibition; (Ox brain)

Summary

A steady-state kinetic investigation of the effect of K^+ on the Na^+-enzyme activity of the ($Na^+ + K^+$)-ATPase in broken membrane preparations is reported. Analysis of the kinetic patterns obtained, together with the results reported in the first two articles of this series permit the following conclusions.

1. K^+ inhibits the Na^+-enzyme (the enzyme activity measured at micromolar substrate concentrations in the presence of Na^+). The inhibition is non-competitive at low and competitive at higher K^+ concentrations and is enhanced by free Mg^{2+}.

2. The results indicate that the Na^+-enzyme at steady-state tends to be accumulated in an enzyme-potassium complex when K^+ is added.

3. The enzyme-potassium complex, in turn, binds Mg^{2+} in a dead-end fashion. The dissociation constant for the enzyme-K-Mg complex, estimated from the data, is 7.2 mM. The same value was obtained earlier for the Mg^{2+} inhibition constant of the substrate-free form of the ($Na^+ + K^+$)-enzyme (the enzyme activity measured with Na^+ and K^+ and at millimolar substrate concentrations) suggesting that the two constants describe the same equilibrium.

4. On the basis of the known (optimal) activity of the ($Na^+ + K^+$)-ATPase, relative to that of the Na^+-ATPase, a rate constant condition is found which must be met if the Post-Albers kinetic scheme is to satisfy the data. Kinetic data for the phosphoenzyme indicate that this condition is not satisfied.

5. On the basis of the kinetic results a model for the hydrolytic action of ($Na^+ + K^+$)-ATPase is proposed. This model encompasses the Post-Albers scheme

but contains two distinctive hydrolysis cycles (an 'Na^+-enzyme cycle' and a '($Na^+ + K^+$)-enzyme cycle') with widely different affinities for the substrates. Only one of the cycles (the Na^+-enzyme cycle) involves acid-stable phosphorylated enzyme intermediates at discernible steady-state concentrations. Which of the two main cycles is predominant in any particular system is determined by the concentrations of ligands and substrates.

6. According to this scheme, an enzyme preparation may exhibit both a high (Na^+-enzyme) and a low (($Na^+ + K^+$)-enzyme) substrate affinity, without the necessity of assigning more than one substrate site to a particular enzyme unit at any one time.

Introduction

In paper I of this series [1], a steady-state kinetic study of ($Na^+ + K^+$)-ATPase from bovine brain was presented. We defined there the Na^+-enzyme as the enzyme activity measured in the presence of Na^+ and at micromolar substrate concentrations, and the ($Na^+ + K^+$)-enzyme as the activity measured with Na^+ and K^+ present and with millimolar substrate concentrations. It was shown that for the two enzymes the data were compatible with (almost) identical kinetic schemes, in which MgATP as well as ($ATP_{free} + Mg^{2+}_{free}$) were substrates, the distinction being that the Na^+-enzyme scheme contained only one dead-end Mg complex while that of the ($Na^+ + K^+$)-enzyme contained two. The two schemes could be characterized by complete sets of kinetic constants, but the two sets are very different. Notably, while the Na^+-enzyme has a high affinity for ATP (measured by the dissociation constant of the E-ATP complex), and a rather low maximal velocity, the ($Na^+ + K^+$)-enzyme is characterized by a low affinity for ATP and a large maximal velocity (see Ref. 1 for details).

Since ($Na^+ + K^+$)-ATPase is generally presumed to be a single enzyme, the symbols denoting corresponding enzyme intermediates in the two schemes must represent different states of the same protein in the two cases.

With the aim of combining the two schemes into a single model, we present a steady-state kinetic study of the Mg^{2+}-enhanced K^+ inhibition of the Na^+-enzyme which provides a clue as to how such a combination may be effected.

The possibility of interpreting the results obtained here and in the two previous papers of this series [1,2] in terms of the Post-Albers scheme [3,4] was investigated, and it is concluded that this scheme, even including the more elaborate version proposed by Karlish et al. [5], is inadequate.

On the basis of our results we propose a new model for ($Na^+ + K^+$)-ATPase which is different from, but contains, the Post-Albers scheme. The most important property of our model is the existence of two different hydrolytic cycles, only one of which (the Na^+-enzyme cycle) contains acid-stable phosphorylated intermediates at appreciable steady-state concentrations.

Materials and Methods

The enzyme preparation and the assay procedures used were as described in paper I [1].

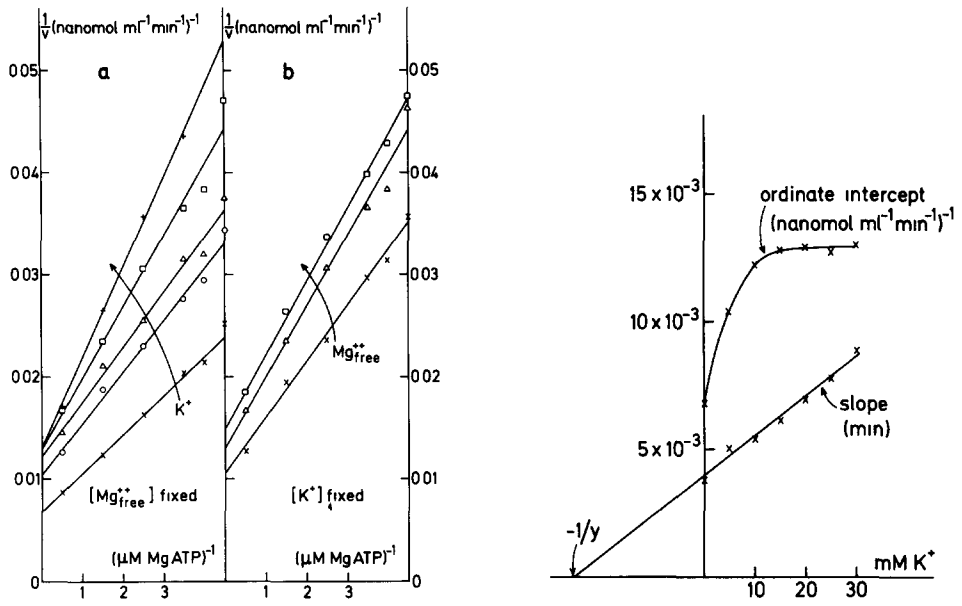

Fig. 1. K^+ inhibition of the Na^+-enzyme. $[Na^+]$ = 150 mM; 30 mM histidine, pH 7.4, 37°C. (a) $[Mg^{2+}_{free}]$ constant (= 12 mM), $[K^+]$ varied: 0 mM (X); 5 mM (○); 10 mM (△); 20 mM (□); 30 mM (+). (b) Enhancement by Mg^{2+} of the K^+ inhibition. $[K^+]$ constant (= 20 mM); Mg^{2+}_{free} concentrations: 6 mM (X); 12 mM (△); 15 mM (□). Rates have been normalized to correspond to undiluted enzyme. The lines drawn are the least-squares regression lines.

Fig. 2. The slopes and ordinate intercepts from lines as in Fig. 1a vs. $[K^+]$. $[Mg^{2+}_{free}]$ constant (= 12 mM). Determination of the quantity y (see text) is indicated. The line for the slope is the least-squares regression line. The curve for the intercept is drawn through the points as 'an aid to the eye'.

Results

Double-reciprocal plots of the data from a series of kinetic runs with: (a) constant $[Mg^{2+}]$ and varying $[K^+]$ and (b) constant $[K^+]$ and varying $[Mg^{2+}]$ are presented in Fig. 1 (for ligand concentrations, see legend). Intersecting straight lines are obtained in all cases, indicating inhibition by K^+ of the Na^+-enzyme. However, the inhibition is not linear * as seen from the secondary plots of slopes, S, and ordinate intercepts, I, from Fig. 1a as a function of $[K^+]$, presented in Fig. 2. The K^+ inhibition is non-competitive at low and competitive at higher K^+ concentrations.

Our primary purpose here is not a complete, quantitative, interpretation of the results. Rather, we shall use the patterns obtained (and the estimate of a single kinetic constant, see below) to obtain a clue regarding the connection between the Na^+-enzyme and the $(Na^+ + K^+)$-enzyme activity.

* We use enzyme kinetic terms as defined by Cleland [6]: Linear non-competitive inhibition by a ligand indicates that the slope and (vertical) intercept of the primary plot are linear functions of the concentration of the inhibiting species. For linear competitive inhibition the slope is a linear function of the inhibitor concentration.

We shall interprete the results on the basis of the mechanism for the Na^+-enzyme obtained previously [1]. As discussed in paper II of this series [2], the Na^+-enzyme scheme should include two phosphorylated intermediates (Fig. 3a). This will of course in general complicate the rate equation, relative to that obtained with only one (phosphorylated) intermediate (as used in paper I). However, at zero K^+ and ADP concentrations, E_1P and E_2P may be considered equivalent in the sense that the form of the rate equation is unchanged, but the kinetic constants are more complex functions of the rate constants. This complexity disappears if, as indicated by the experiments reported in paper II at 0°C, the dephosphorylation rate constants of E_1P and E_2P are approximately equal. If this is true also at 37°C, it may be shown that the rate equation with two phosphorylated intermediates is identical with that obtained in paper I [1] with only one intermediate.

When K^+ is present in the medium this equivalence between E_1P and E_2P no longer applies, since only E_2P (see Discussion) is sensitive to K^+. Both intermediates must therefore be included in the analysis of K^+ inhibition.

We can make one simplification in the model in Fig. 3a, however, in view of the values of some of the kinetic parameters: We found in paper I that for $[Mg^{2+}] > 3$ mM, less than 10% of the total net reaction flux is due to the cycle $E_1 \rightarrow E_1A \rightarrow E_1MA \rightarrow (E_1P + E_2P) \rightarrow E_1$. Hence, under these conditions, we can without serious error omit the enzyme intermediate E_1A, to obtain the model in Fig. 3b.

A simple way for a ligand to result in a non-competitive effect is for it to interact with at least two enzyme forms in the mechanism, one of which is the substrate-free enzyme form. Since in our case, as seen from Fig. 1b, the observed K^+ inhibition is further enhanced by free Mg^{2+}, the simple scheme shown in Fig. 3c is suggested. In this scheme, K^+ is not a dead-end inhibitor, but the form EK is a part of a hydrolysis cycle. To observe inhibition we would expect $k_4 \cdot [K^+] > k_3$ and k_5, resulting in the accumulation of the enzyme in the states EK and MEK during hydrolysis. That the first of these inequalities may indeed be expected to hold is suggested by several experiments on the dependence of the dephosphorylation rate on potassium [4,7–9].

In order to see whether the mechanism in Fig. 3c may represent the data obtained we must derive the steady-state rate equation and investigate its proper-

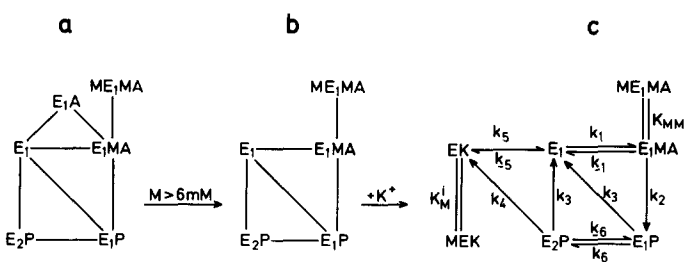

Fig. 3. (a) The kinetic model for the Na^+-enzyme, derived in paper I. (b) The essential part of the model in a when 6 mM $\leq [Mg^{2+}_{free}]$ (see text). (c) The model proposed for the K^+ inhibition of the Na^+-enzyme, showing rate constant assignments. Double lines indicate equilibrium characterized by the associated dissociation constant.

ties. Using the procedure of King and Altman [10] we obtain for the reciprocal rate (MA, K, M are the concentrations of MgATP, K^+, and free Mg^{2+}, respectively):

$$\frac{1}{v} = \frac{K_m^{app}}{V_m^{app}} \cdot \frac{1}{MA} + \frac{1}{V_m^{app}} \tag{1}$$

where the kinetic parameters K_m^{app} and V_m^{app} are complex functions of K, M, and the rate constants. For the ordinate intercept $1/V_m^{app}$ we obtain a function of the form:

$$\frac{1}{V_m^{app}} = \frac{1}{V_{MA}} \frac{a(M) + b(M) \cdot K}{1 + K/K_K^i}; \quad K_K^i \equiv \frac{k_3}{k_4} \tag{2}$$

where V_{MA} is V for the Na^+-enzyme, determined in paper I, and $a(M)$ and $b(M)$ are functions of the rate constants and M. We note from Eqn. 2 that when K is increased, a plateau for the intercept is attained, in accordance with the data (Fig. 2). The plateau value depends on M in a linear fashion (not shown).

For the slope $S = K_m^{app}/V_m^{app}$ we obtain

$$S = \frac{K_{MA}}{V_{MA}} \left[1 + \frac{K}{K_K^e}\left(1 + \frac{M}{K_M^i}\right)\right] \tag{3}$$

where

$$K_K^e = \frac{k_5}{k_{-5}} \tag{4}$$

and K_{MA} is the Michaelis constant for MgATP for the Na^+-enzyme, determined in paper I. The linear relationship, Eqn. 3, is in agreement with the data (Fig. 2). Eqn. 3 permits the estimation of K_M^i: Considering S as a function of K at various, fixed, M values we note that the reciprocal abscissa intercepts, y, (indicated on Fig. 2) are:

$$-y = \frac{1}{K_K^e} + \frac{M}{K_K^e K_M^i} \tag{5}$$

From the slope and (ordinate) intercept of a graph of $|y|$ vs. M, we then obtain K_M^i. Such a graph, determined from slope lines as in Fig. 2, is shown in Fig. 4, from which we obtain

$$K_M^i = 7.2 \text{ mM} \tag{6}$$

$$K_K^e = 66 \text{ mM} \tag{7}$$

It thus appears that the simple scheme (Fig. 3c) yields a rate equation sufficiently complex to account for the patterns obtained from experiments and exhibited in Figs. 1, 2 and 4. In this scheme K^+, while being an effective dephosphorylating agent, leads to an intermediate, EK, of the enzyme, the conversion of which to E_1 becomes rate limiting. In addition, the trapping of the enzyme is enhanced by free Mg^{2+} which inhibits the EK form in a dead-end fashion.

We do not attempt here an estimation of the remaining parameters in Eqns.

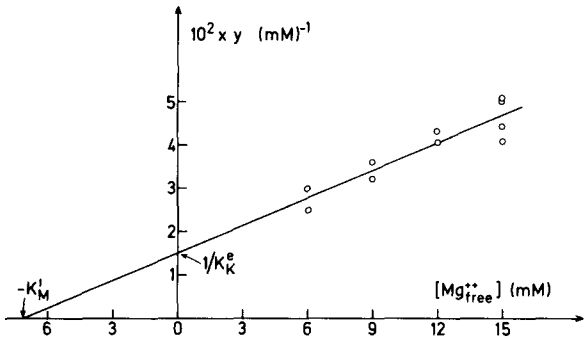

Fig. 4. Determination of the kinetic constants K_M^i and K_K^e. The quantity y (see text) (from lines as in Fig. 2) vs. $[Mg^{2+}_{free}]$. From the regression line is found: $K_M^i = 7.2$ mM; $K_K^e = 66$ mM.

2 and 3. Such a determination as well as the possibility of estimating individual rate constants in the mechanism is presently being explored, but is unnecessary for our present purpose.

Discussion

It is natural to ask whether the results reported here, as well as those obtained and discussed in papers I and II of this series [1,2], are compatible with the recent versions of the Post-Albers scheme [5,7] for ($Na^+ + K^+$)-ATPase, since this model rests on a considerable body of experimental evidence and serves as frame of reference for most studies on ($Na^+ + K^+$)-ATPase (for reviews see Refs. 11 and 12).

From the kinetic data in paper I we note the rather different sets of kinetic constants for the Na^+-enzyme and the ($Na^+ + K^+$)-enzyme. In particular, the maximal velocity of the ($Na^+ + K^+$)-enzyme is at least 25-times that of the Na^+-enzyme (see also Refs. 13—15). We therefore inquire whether a kinetic scheme, such as the Post-Albers model, in which a substantial part of the scheme is common for the Na^+ and the ($Na^+ + K$) activity, is compatible with this observation.

In the Post-Albers scheme the sequence *:

$$\rightarrow E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \dashrightarrow E_2K \qquad (I)$$
$$\qquad\qquad\quad \searrow^{k_3}$$
$$\qquad\qquad\quad E_2 + P_i$$

leading from E_1P to E_2P must be traversed whether or not K^+ is present. In the Appendix we show that the observed ratio (approx. 25) between the maximal velocities with and without K^+ requires:

$$k_3 << k_{-2} \qquad (8)$$

* Note that, for ease of comparison of the present discussion with the arguments in paper II, the rate constants governing the interconversion $E_1P \rightleftharpoons E_2P$ are here denoted k_2 and k_{-2} and not, as in Fig. 3c, k_6 and k_{-6}.

But from the experimental work reported in paper II we conclude that:

$$k_3 > 10\, k_{-2} \tag{9}$$

and from the discussion presented there (cf. also below) it is seen that a similar relationship between k_3 and k_{-2} is apparent from the work of others in this field, at least up to 21°C. Thus, while it is theoretically possible (namely if requirement 8 were fulfilled) for the Post-Albers scheme to exhibit the observed ratio of maximal velocities, the experimental observations (Eqn. 9) rule out this possibility. A model in which the Na^+-ATPase and ($Na^+ + K^+$)-ATPase activity have in common the step containing the two phosphorylated intermediates must therefore be considered incompatible with the observed difference in V with and without K^+ in the medium.

The applicability of the Post-Albers scheme in explaining the K^+ dependence of ATP hydrolysis by ($Na^+ + K^+$)-ATPase from ox brain has been examined by Mårdh and his colleagues [8,16,17] in a series of papers. Since they apparently arrive at a conclusion opposite to that above in Eqn. 9, we shall discuss their work briefly in the following. By a rapid mixing technique the time course of phosphorylation and dephosphorylation at 21°C was monitored and some of the results were evaluated in terms of rate constants* for the partial reactions including those involved in the turnover of E_1P and E_2P

$$\xleftarrow[k_{-1}]{} E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \xrightarrow[k_3]{} \tag{II}$$

As outlined in paper II, we can extract from the results of Mårdh [8] on the dephosphorylation rate at 21°C in the absence of K^+ that k_3 ($\approx$150—200 min^{-1}) is considerably larger than k_{-2}, and that k_2 is 400—500 min^{-1}. The fact that $k_{-2} < k_3$ supports our observation at 0°C and argues against scheme II as part of the ($Na^+ + K^+$)-ATPase cycle also under the conditions used by Mårdh.

However, in their subsequent simulation study, Mårdh and Lindahl [17] use a calculated value of $k_{-2} = 1800$ min^{-1}, about 10-times k_3, and this enables them to obtain reasonable fits to phosphorylation data from experiments both without and with K^+ in the medium. Mårdh and Lindahl [17] calculate k_{-2} from $k_2 = 4600$ min^{-1} and 'the equilibrium between E_1P and E_2P in the absence of K^+', implying that k_{-2} and k_2 are not affected by potassium *.

The value of 4600 min^{-1} for k_2 is clearly crucial in the argument in favor of the Post-Albers scheme, since it is just large enough to satisfy the criterion that $[E_1P] \times k_2 = v$ for ATP hydrolysis in the presence of 10 mM K^+ (Ref. 8, p. 458) (the back reaction k_{-2} $[E_2P]$ can be neglected here because $[E_2P]$ is very low). This value of k_2 stems from an experiment in which a direct determination of k_2 was attempted by monitoring the dephosphorylation rate in the presence of 10 mM K^+ (Ref. 8, Figs. 3B and C, and 5). According to scheme II, E_2P is either absent or disappears very rapidly by addition of K^+ since k_3 in the presence of K^+ is at least 14 000 min^{-1} [8,16]. Determination of the rate constant for the dephosphorylation of the remaining 25% of EP_{total} corresponding to E_1P is ob-

* From the data [9] and analysis [2] by Klodos et al. it appears that $k_3 > k_{-2}$, and that k_2 is constant, also in the presence of stimulating concentrations of K^+ or Li^+.

viously difficult and Mårdh arrives at three different values: 1700, 2800 and 4600 min^{-1}. In discussing the possible reasons for the discrepancy between the value of 400—500 min^{-1} for k_2 calculated by us from Mårdh's data (see above) and that of 4600 used by Mårdh and Lindahl, we find it important to emphasize the following points (referring to Ref. 8):

1. Determination of the rate of dephosphorylation of E_1P is experimentally difficult since the difference $[EP]_t - [EP]_{\infty app}$ is small (Figs. 3 and 5). A small variation in $[EP]_{\infty app}$ will seriously influence the slope of the curves.

2. The justification for choosing the value 4600 min^{-1} out of the three values 1700, 2800 and 4600 min^{-1} is not clear (Ref. 8, p. 458).

3. The rate constant from Fig. 5 is $k_2 + k_{-1}$ and not k_2. In the simulation study [17] a value of 2400 min^{-1} for k_{-1} in the absence of added ADP is necessary to obtain a good fit. This value may be even greater in the ATP experiment of Fig. 5, which gives the $k_2 + k_{-1}$ value of 4600 min^{-1}, because the unlabelled ATP used to stop the phosphorylation may be contaminated with ADP. Furthermore, spontaneous dephosphorylation of E_1P to E_1 and P_i for which we have presented evidence [2], will contribute to the difference between the measured constant and k_2.

Before concluding this part of the discussion we shall stress that the rephosphorylation experiment (Ref. 8, Fig. 8B) quoted by Mårdh to be in agreement with his hypothesis is inconclusive. The calculation of EP turnover assumes that steady-state with regard to [EP] was present even if [ATP] was increased from 5 to 100 μM. That this is not the case is demonstrated by Fig. 4 in the paper by Mårdh and Zetterquist [16] showing that [EP] is increased by a factor of 3 by such an increase in the ATP concentration.

With the arguments presented above on the role of E_1P and E_2P in the $(Na^+ + K^+)$-ATPase reaction we find that the evidence is against a model with the step $E_1P \rightarrow E_2P$ as a crucial step.

A way to extend the Post-Albers scheme such that the maximal velocity relationship is accounted for is suggested by the experiments on K^+ inhibition reported here. The equality of the kinetic constants K_M^i (= 7.2 mM), estimated from the Mg^{2+}-enhanced K^+ inhibition of the Na^+-enzyme (Fig. 3c), and the previously obtained constant K_{OM} (= 7.6 mM) [1] describing Mg^{2+} inhibition of the substrate-free form of the $(Na^+ + K^+)$-enzyme, suggests that the two constants describe the same equilibrium, and that the form EK (Fig. 3c) may thus be identified with the intermediate of the $(Na^+ + K^+)$-enzyme to which the substrates add. These considerations lead us to propose the model shown in Fig. 5 as the simplest scheme compatible with the evidence presented here and in papers I and II.

In the present discussion we conform to common usage and denote by E_1 and E_2 the two conformational states of the $(Na^+ + K^+)$-ATPase usually associated with, respectively, the 'sodium-form' and the 'potassium-form'. We do not with this notation necessarily imply any commitment regarding the 'sidedness' of the two states. Note also that in the enzyme intermediates in the left-hand cycle in Fig. 5 appearing upon addition of substrates, the conformational state of the enzyme protein is left unspecified.

The Post-Albers scheme is contained in Fig. 5: $E_1 - E_1MA - E_1P - E_2P - E_2K - E_1$. In addition, we propose that E_2K is also able to hydrolyze ATP, with

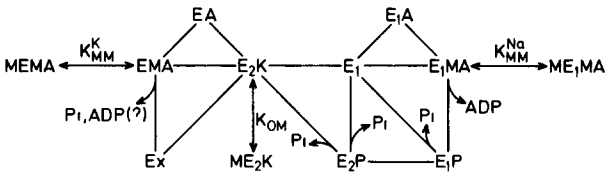

Fig. 5. The proposed minimal kinetic model for the hydrolytic action of $(Na^+ + K^+)$-ATPase, showing two main hydrolysis cycles: $E_1 - (E_1A) - E_1MA - E_1P - E_2P - E_1$ (the Na^+-enzyme), and $E_2K - (EA) - EMA - E_x - E_2K$ (the $(Na^+ + K^+)$-enzyme).

a low substrate affinity and a large maximal velocity, represented by the cycle(s) $E_2K - (EA) - EMA - E_x - E_2K$.

In terms of our model, the interpretation of the observed K^+ inhibition of the Na^+-enzyme is that, despite the appearance when adding K^+ to the Na^+-enzyme, of the form E_2K which also possesses the ability to hydrolyze ATP, this ability is not manifested at the low (micrmolar) substrate concentrations used.

In the right-hand cycles $E_1 - (E_1A) - E_1MA - E_1P - E_2P - E_1$ of the scheme in Fig. 5, representing the action of the Na^+-enzyme, we have assumed the forms E_1P (sensitive to ADP) and E_2P (insentive to ADP but sensitive to K^+) to be able to undergo spontaneous dephosphorylation to yield the empty enzyme form E_1, in accordance with the evidence discussed in paper II.

The left-hand cycles $E_2K - (EA) - EMA - E_x - E_2K$ represent the $(Na^+ + K^+)$-enzyme which is active at higher substrate concentrations. The 'empty enzyme' is E_2K which can bind Mg^{2+} to form a dead-end complex and which has only a low-affinity site for substrates. The intermediate corresponding to $(E_1P + E_2P)$ for the Na^+-enzyme (Fig. 3a) is denoted E_x to indicate its unspecified nature. We submit that E_x is not a phosphorylated enzyme intermediate or, alternatively, if it is, it is acid labile. In neither case would a phosphorylated intermediate be experimentally observable at steady state (using current methods) if only the left-hand cycles are taken into account.

It was mentioned in paper I that an intermediate, such as E_x, between EMA and the 'empty' enzyme in the hydrolysis mechanism is not necessary to explain the functional form of the kinetic data; its omission would merely simplify the expressions for the kinetic constants involved. While the existence of such forms for the Na^+-enzyme (E_1P and E_2P) is amply supported by experiments, this is not the case for the new cycle proposed here for the potassium form. However, it may be shown that the implications resulting from identifying the enzyme-substrate complexes EMA, obtained from addition of substrates along the two alternative pathways ($E_2K - EMA$ and $E_2K - EA - EMA$), in conjunction with the values of some of the kinetic constants for the $(Na^+ + K^+)$-enzyme determined in paper I, make it mandatory that at least one intermediate E_x exists. A detailed account of this analysis will appear in a future publication.

It has been shown [18] that incubating the enzyme with K^+ and (large, 5 mM) ATP concentrations yields a trypsin digestion pattern for the enzyme identical to that obtained with only Na^+ present in the incubation medium. From

this result it might be argued that the intermediate EA in Fig. 5 should belong to the 'E_1 family' of conformations. That this is not necessarily so may be seen as follows: in terms of our model (Fig. 5) when only K^+ and ATP are present, the following intermediates should exist:

$$EA \overset{K_1}{\rightleftharpoons} E_2K \overset{K_2}{\rightleftharpoons} E_1 \overset{K_3}{\rightleftharpoons} E_1A \qquad \text{(III)}$$

Under the conditions of the cited experiments no hydrolysis occurs, and we can assume scheme III to represent an equilibrium situation, characterized by the (dissociation) constants indicated. From paper I and the present work the constants are:

$K_1 \equiv K_{iA}^{(K)} \cong 200\ \mu M$

$K_2 \equiv K_K^e = 66 \cdot 10^3\ \mu M$

$K_3 \equiv K_{iA}^{(Na)} \cong 0.8\ \mu M$

The ratio $\rho = (E_1 + E_1A)/(E_2K + EA)$ is easily found to be

$$\rho = \frac{\frac{K_2}{K}\left(\frac{K_1}{K_3} + \frac{K_1}{A}\right)}{1 + \frac{K_1}{A}}$$

where K and A are K^+ and ATP concentrations, respectively. For $K = 100$ mM, $A = 3$ mM we obtain: $\rho \simeq 160$, and thus less than 1% of the enzyme is present as the E_2 conformation (assuming that EA and E_2K have similar conformations). Under these circumstances, the latter would not be observable, and hence EA need not belong to the E_1 class.

This argument does not preclude that most of the enzyme, when hydrolysis is allowed to proceed at large substrate concentrations, is present as one or more of the potassium forms (i.e., in the left-hand cycle in Fig. 5).

The existence of a direct path between E_1 and E_2K is in accordance with the work of Karlish et al. [5]. Their results indicate that for the transition $E_1 \rightarrow E_2K$ the observed rate constant is roughly proportional to $[K^+]$ (at least up to 15 mM K^+). This seems consistent with Fig. 5.

It should be noted that according to the model (Fig. 5) a particular enzyme molecule has, at any one time, only one substrate site, of which the affinity for the substrate(s) is determined by the (conformational) state of the protein. Had K^+ acted on the Na^+-enzyme by opening up or creating a new substrate site, in addition to the high-affinity site already present, then K^+ would have had an activating or at the least no effect.

Of course, in an enzyme preparation working at steady state in the reaction medium, all the enzyme intermediates represented in Fig. 5 are present in amounts depending upon the composition of the medium. The concentrations of the various ligands will also determine the fraction of the total reaction flux associated with a particular cycle (an example of such a calculation for a part of the model was presented in paper I of this series). At the usual cellular concentrations of Na^+, K^+, Mg^{2+} and ATP, we could expect the main flux to be associated with the ($Na^+ + K^+$)-enzyme (the left half of the model in Fig. 5), which, as mentioned above, does not involve an acid-stable phosphorylated

intermediate. In this sense, the pathway involving the intermediates E_1P and E_2P is of minor importance, and the partial reactions in that pathway may be deceptive, in relation to the working of the pump under physiological conditions, as has also been suggested by others [19,20,24].

The existence for this enzyme of two different hydrolysis cycles, one of which does not involve phosphorylated intermediates, has been suggested several times by Skou [19,20]. It is alsso in accord with conclusions drawn by Brodsky and coworkers [21—23] and with those of Whittam and Chipperfield [24].

The proposed model, as it stands, also seems able, at least qualitatively, to explain the observation by Klodos and Nørby [9] that the rate of dephosphorylation of the phosphorylated enzyme is not sufficient to account for the total hydrolytic activity when K^+ or (especially) Li^+ is present.

We emphasize that since we have not, in this work, been concerned with the detailed kinetic action of Na^+ and K^+, we have omitted explicit indication of their presence on the enzyme forms in Fig. 5, except for the form E_2K. This should not, of course, be taken to imply that we believe the latter to be the only form to which a cation is bound. Several, or possibly all, of the enzyme forms probably contain one or more cations, as they must if transport across the membrane is to be accomplished.

Clearly, the ultimate interest in the kinetic mechanism for $(Na^+ + K^+)$-ATPase lies in its usefulness as a foundation for such a transport scheme for the system in intact membranes. For this usage it will be necessary to associate ion translocation with several steps in the kinetic model. How this should be done, in view of the evidence at hand, and whether the various modes of the sodium pump could be explained on the basis of our proposed model is presently under study.

Appendix

We derive here a rate constant condition which must be satisfied, if a model in which a particular step must necessarily be traversed be able to exhibit the observed ratio of the maximal activities with and without K^+ in the medium.

We consider the experimentally established sequence:

$$\rightarrow E_1P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2P \begin{cases} \nearrow (E_2K) \\ \overset{k_3}{\searrow} \text{spontaneous dephosphorylation} \end{cases}$$

At steady state, the net fluxes through the step $E_1P - E_2P$ are:

$$J(K = 0) \equiv J_0 = k_2 \epsilon_1^o - k_{-2} \epsilon_2^o \tag{A1}$$

$$J(K \neq 0) \equiv J_K = k_2 \epsilon_1^K - k_{-2} \epsilon_2^K \tag{A2}$$

where ϵ_i^o and ϵ_i^K are the steady-state concentrations of E_iP without and with K^+ in the medium, respectively, and k_2 and k_{-2} (independent of K^+) are the rate constants governing the interconversion. We set:

$$\begin{aligned} \epsilon_1^K &\equiv \alpha_1 \epsilon_1^o \\ \epsilon_2^K &\equiv \alpha_2 \epsilon_2^o \end{aligned} \tag{A3}$$

in Eqns. A1 and A2, and since, under appropriate optimal cation concentrations, we have $J_K \simeq 25\, J_0$ [13—15], we obtain:

$$k_2 \epsilon_1^o \left(1 - \frac{\alpha_1}{25}\right) = k_{-2} \epsilon_2^o \left(1 - \frac{\alpha_2}{25}\right). \tag{A4}$$

At steady state, with no K^+ in the medium, we have:

$$\frac{d\epsilon_2^o}{dt} = 0 = k_2 \epsilon_1^o - (k_{-2} + k_3)\, \epsilon_2^o \tag{A5}$$

whence:

$$k_2 \epsilon_1^o = (k_{-2} + k_3)\, \epsilon_2^o\ . \tag{A6}$$

From Eqn. A4 we obtain, using Eqn. A6:

$$(k_{-2} + k_3)\left(1 - \frac{\alpha_1}{25}\right) = k_{-2}\left(1 - \frac{\alpha_2}{25}\right) \tag{A7}$$

which, since $\epsilon_1^K \leqslant \epsilon_1^0$ [8,9], i.e., $\alpha_1 \leqslant 1$, and $\epsilon_2^K < \epsilon_2^0$ [8,9], i.e., $\alpha_2 < 1$, leads to:

$$k_3 << k_{-2} \tag{A8}$$

as the necessary condition for the transition $E_1P \rightarrow E_2P$ to be an essential step in the hydrolytic action of the enzyme also when K^+ is present in the medium.

Acknowledgements

We are grateful to J.C. Skou for several illuminating discussions. Mrs. Vinni Ravn is thanked for her careful technical assistance. This work was supported by a grant from the Danish Medical Research Council, J. No. 512-10806.

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