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Distinction between the intermediates in Na$^+$-ATPase and Na$^+$,K$^+$-ATPase reactions. I. Exchange and hydrolysis kinetics at millimolar nucleotide concentrations

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Parallel measurements in steady-state of ATP hydrolysis rate (v_{hydr}) and the simultaneous reverse reaction, i.e., the ADP–ATP exchange rate (v_{exch}), allowed the determination of a kinetic parameter, K_E, containing only the four rate constants needed to characterize the enzyme intermediates involved in the sequence

$$\xleftarrow{-1} \mathrm{EATP} \underset{-2}{\overset{2}{\rightleftarrows}} \mathrm{ADP} + \mathrm{EP} \xrightarrow{3}$$

$$K_E = v_{exch}/(v_{hydr}[\mathrm{ADP}]) = \frac{k_{-2}}{k_3(1 + k_2/k_{-1})}$$

In order to compare the properties of these enzyme intermediates under different sets of conditions, K_E was measured at varying K$^+$ and Na$^+$ concentrations in the presence of millimolar concentrations of ATP, ADP and MgATP, using an enzyme preparation that was partially purified from bovine brain. (1) In the presence of Na$^+$ (150 mM), K$^+$ (20–150 mM) was found to increase the exchange rate and decrease the ATP hydrolysis rate at steady-state. As a result, K_E increased at increasing K$^+$. However, the value of K_E found by extrapolation to K$^+$ = 0 was 7-times lower than the value actually measured in the absence of K$^+$. This finding indicates that one of the intermediates, EATP or EP, or both, when formed in the presence of Na$^+$ alone, are different from the corresponding intermediate(s) formed in the presence of Na$^+$ + K$^+$ (at millimolar substrate concentration). (2) In the presence of 150 mM K$^+$, Na$^+$ (5–30 mM) was found to increase the ADP/ATP exchange as well as the ATP hydrolysis rate at steady-state. The ratio of the two rates was constant. This finding, when interpreted in terms of K_E, indicates that Na$^+$ does not have to leave the enzyme for ATP release to be accelerated by K$^+$ in the backward reaction. This also is in opposition to the usual versions of the Albers-Post model, which does not have simultaneous presence of Na$^+$ and K$^+$.

Introduction

The ouabain-sensitive, (Na$^+$ + K$^+$)-activated ATPase (EC 3.6.1.37) is the enzymatic activity identified with the coupled transport of Na$^+$ and

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K^+ across the cell membrane [1]. According to the most generally accepted scheme, the Albert-Post scheme [2,3], for the action of the enzyme, Na^+ and K^+ are consecutively transported during the hydrolysis of ATP, which proceeds through the same series of phosphorylated intermediates, whether the substrate concentration is high or low and whether K^+ is present or not.

From time to time, the validity of the Albert-Post scheme has been disputed [4–8] and, recently, in a series of papers [9–17] the experimental results presented seemed to show that the phosphorylated enzyme intermediates formed in the presence of ATP and Na^+ alone are different from those that will be formed when also K^+ is present, if the substrate concentration is high, i.e., under physiological conditions. (It was not questioned that hydrolysis by the Na^+ enzyme (i.e., activity measured in the presence of Na^+ alone and that measured at micromolar substrate in the presence or in the absence of K^+) proceeds through the well-known phosphorylated intermediates of the Albers-Post scheme.)

The evidence may be summarized as follows:

(1) Steady-state kinetic investigations [11,15–17] allowed an estimation of the intrinsic (i.e., ligand-independent) rate constant for the step in which ADP, the first product, is released from the enzyme. The rate constant obtained from hydrolysis rates measured in the presence of millimolar concentrations of ATP and $Na^+ + K^+$ was approx. 30-times higher than the rate constant calculated corresponding to Na^+-ATPase conditions. This could not be obtained if the intermediate that splits into ADP and phosphoenzyme is the same under the two sets of conditions, as in the Albers-Post scheme.

(2) From steady-state kinetic investigations [14,15,17] it was concluded that the intermediate that binds substrate at physiological conditions, i.e., at millimolar concentrations of ATP and in the presence of $Na^+ + K^+$, most probably has K^+ and Na^+ bound to it simultaneously. The same conclusion has been reached by others [18,6,45], and it is inconsistent with a wholly consecutive transport mechanism.

(3) It has repeatedly been found [8,12,19,20] that the enzyme activity measured in the presence of millimolar ATP concentrations and $Na^+ + K^+$ exceeds the ATP hydrolysis rate calculated on the basis of dephosphorylation rates measured when K^+ was present only during dephosphorylation, but not during phosphorylation. Through a detailed transient kinetic investigation [9,10,12,13] of the interconversion of the phosphorylated intermediates formed in the presence of Na^+ alone, it was possible to demonstrate that K^+ actually inhibits the interconversion between the phosphoenzyme first formed and the following intermediate [12,13]. As K^+ increases the hydrolysis rate (at millimolar substrate), the rate corresponding to each step in the hydrolysis cycle should be increased in the presence of K^+. Therefore, the K^+ inhibition of the interconversion supports the view that the phosphorylated intermediates formed in the presence of Na^+ alone are not part of the hydrolysis cycle when also K^+ is present at millimolar substrate.

On this background, we suggested a bicyclic scheme for the reaction mechanism containing a Na^+ATPase cycle and a $(Na^+ + K^+)$-ATPase cycle, doubly connected but sharing no intermediates [10].

It has been the purpose of the work to be reported below, through parallel measurements of ATP-hydrolysis rates and ADP–ATP exchange rates at steady-state, to obtain independent evidence for both points 1 and 2 above. Point 3 is the subject of the following paper [21].

A preliminary account of parts of this work was presented as a poster at the Fifth International Conference on $(Na^+ + K^+)$-ATPase, Denmark, June, 1987.

Materials and Methods

Enzyme. $(Na^+ + K^+)$-ATPase was prepared from ox brain as described by Klodos et al. [22]. The undiluted enzyme preparation contained 3.2 mg protein per ml and its activity was 4.4 μmol ATP hydrolyzed/mg protein per min, measured with 3 mM ATP/130 mM NaCl/20 mM KCl/3 mM $MgCl_2$/30 mM histidine buffer (pH 7.4) at 37°C. Under these conditions less than 1% of the activity was not inhibited by 1 mM G-strophantin (ouabain). The enzyme was about 10% pure and contained the two forms of the catalytic subunit, α and α^+, in nearly equal amounts. (Sweadner, K.J., private communication).

ADP/ATP exchange rate and ATP hydrolysis measurements. These were carried out in parallel, i.e., enzyme dilution and assay concentrations were the same and are given in the legends to figures.

It is necessary in order to maintain steady-state [27] to ensure by dilution of the enzyme that the fastest of the two opposing processes changes by less than 10% the concentration of the species (MgATP or ADP_{free}) that is present in the lowest concentration. For the calculation of the enzyme dilution it was (at high rates of ADP–ATP exchange) necessary to take into account that $[^3H]ADP$ is diluted by unlabeled ADP at a rate that is equal to the sum of the ATP hydrolysis and the ADP–ATP exchange rates.

Assay temperature was 37 ° C and assay time 10 min. The ouabain-insensitive activity was measured as a blank corresponding to each assay tube. The blank contained 1 mM ouabain. To calculate the concentrations of free nucleotides and Mg^{2+}, the following dissociation constants were used: 0.085 and 0.85 mM for MgATP and MgADP, respectively [23,24].

ADP–ATP exchange rate. This was measured according to the method of Randerath and Randerath [25] as modified by Cashel et al. [26]. The assay volume was 160 μl. The reaction was stopped by addition of 30 μl 30% trichloroacetic acid and the solution was neutralized with 55 μl of 1 M NaOH. From the final volume of 245 μl, 100 μl were spotted on a PEI-cellulose sheet. After chromatography, the ATP spot was cut and transferred to a counting vial [27], where elution took place at room temperature with 1.5 ml of 0.7 M $MgCl_2$ in 20 mM Tris-HCl (pH 7.4). After 25 min scintillation liquid was added for counting of $[^3H]ATP$, and counting efficiency was found to be 31–32%.

ATP hydrolysis rate. This was measured according of the method of Lindberg and Ernster [28].

Chemicals. Adenosine 5′-triphosphate, disodium salt, and Adenosine 5′-diphosphate, disodium salt, were from Boehringer, Mannheim. Adenosine 5′-$[^{32}P]$triphosphate, triethylammonium salt, and $[2\text{-}^3H]$adenosine 5′-diphosphate, ammonium salt, were from Amersham (Code Pb

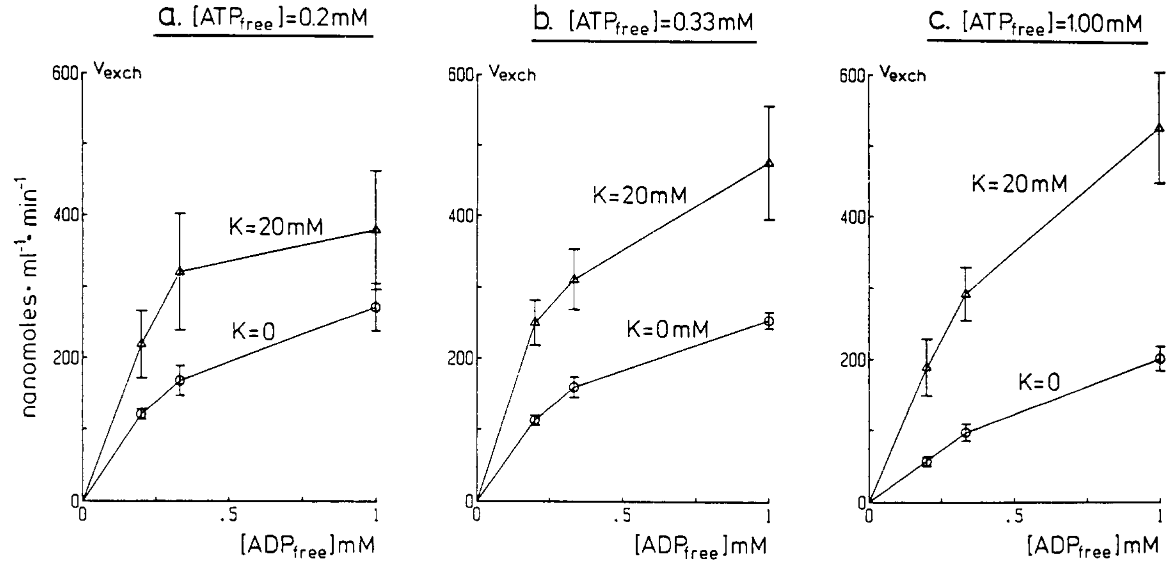

Fig. 1. ADP–ATP exchange rates measured at steady-state versus the concentration of ADP_{free}. The ADP–ATP exchange rate was measured at three different concentrations of ATP_{free} given in the figure, and in the presence as well as in the absence of 20 mM K^+, as indicated. The assay mixture contained 150 mM Na^+, 0.1 mM Mg_{free} and MgATP at 0.235 (a), 0.392 (b) and 1.177 mM (c) in a 30 mM histidine buffer (pH 7.4) at 37 ° C. The rate is given in nmol of ATP formed/min per ml of undiluted enzyme, which contained 3.21 mg protein per ml. The enzyme was diluted 6.4-times in the assay and 500–1000-times before assay when K^+ was present and 50–100-times when K^+ was absent. The specific activity of $(^3H)ADP$ was $3 \cdot 10^4$ to $6 \cdot 10^4$ cpm/nmol. The values are the means of three (without K^+) and five (with K^+) experiments, each containing double determinations. The lengths of the vertical bars are twice the standard deviation.

10132 and TRK. 345, respectively). PEI-cellulose sheets were from Macherey-Nagel (Polygram CEL 300 PEI, No. 801-053).

Results and Discussion

In Figs. 1 and 2 are shown the exchange rate (Fig. 1) and the ATP hydrolysis rate (Fig. 2) at steady-state as a function of the concentration of ADP_{free} at three different concentrations of ATP_{free} with and without K^+ (20 mM).

The ATP hydrolysis rate decreases at increasing concentration of ADP_{free}, and when the reciprocal of the rates given in Fig. 2d–f are plotted against the reciprocal of the substrate (MgATP) concentrations, Fig. 3 is obtained, showing two effects

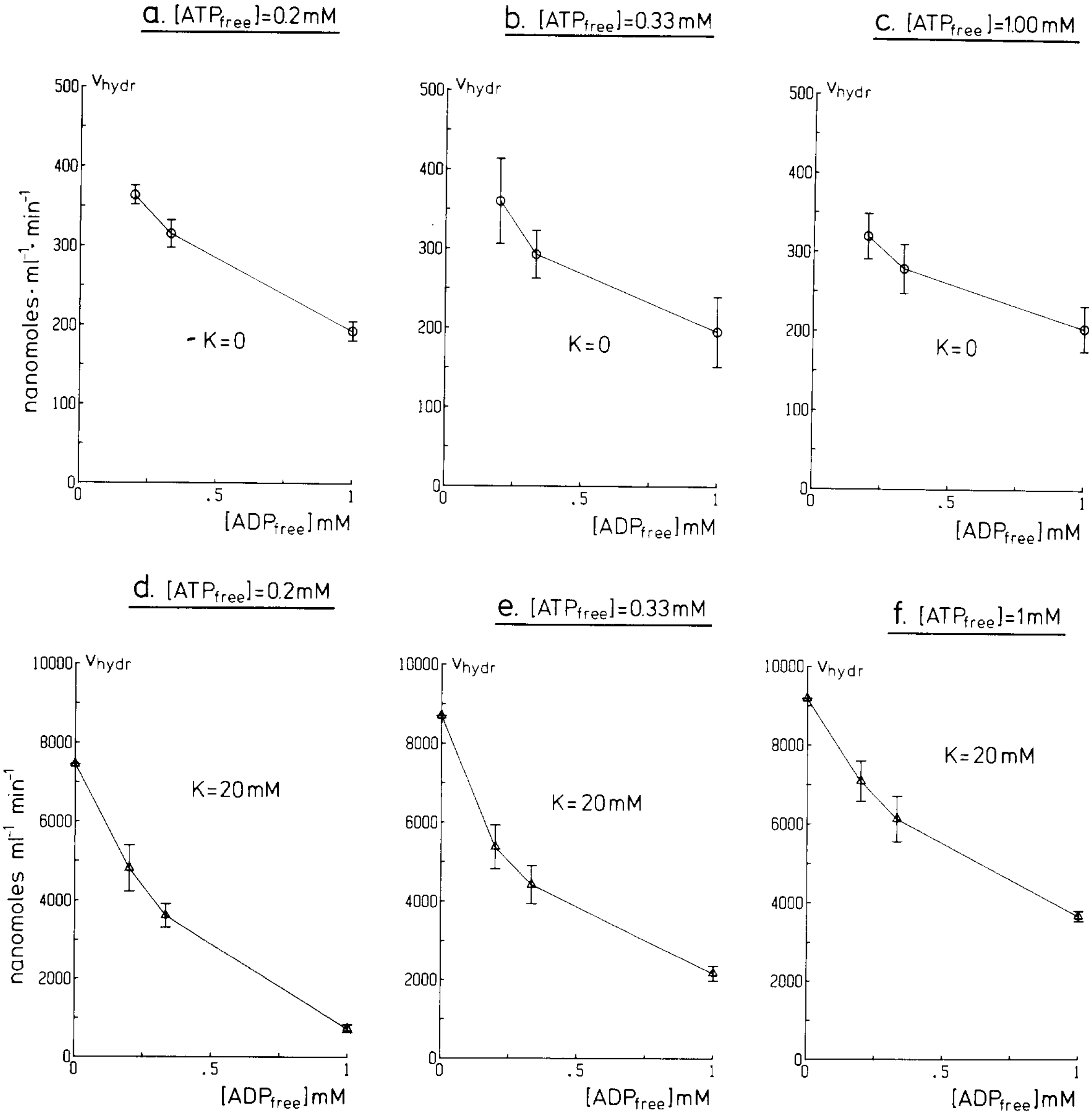

Fig. 2. ATP hydrolysis rates measured at steady-state versus the concentrations of ADP_{free}. Assay conditions are the same as in Fig. 1. The hydrolysis rate was measured at the three different concentrations of ATP_{free} given in the figure and in the presence as well as in the absence of 20 mM K^+ as indicated. The rate is given in nmol of P_i formed/min per ml of undiluted enzyme. The enzyme was diluted as described in Fig. 1. The specific activity of $[^{32}P]ATP$ was $3 \cdot 10^3$–$6 \cdot 10^3$ cpm/nmol. The values are means of three experiments. The lengths of the vertical bars are twice the standard deviation.

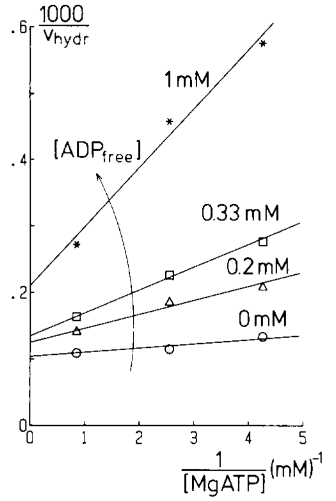

Fig. 3. The reciprocal of the ATP hydrolysis rates given in Fig. 2d–f plotted versus the reciprocal of the substrate (MgATP) concentration.

of ADP on ATP hydrolysis: (1) ADP increases the slope of the double reciprocal plot. This effect was previously reported and interpreted to reveal ADP (or MgADP) competition on the substrate site [29–31]. (2) ADP increases the ordinate intercept. Probably due to the presence of very high Mg^{2+} concentrations in the quoted work [29–31] this effect was not observed. The pattern obtained in Fig. 3 is explained, however, if ADP (and not MgADP) is released from the enzyme [32], and if ADP is the first product of the reaction.

No double reciprocal representation is given of the hydrolysis rates measured in the absence of K^+ corresponding to Fig. 2a–c, as this substrate concentration range in the absence of K^+ is saturating. In addition, substrate inhibition occurs (see following paper) [21].

The exchange rate is an increasing, apparently hyperbolic function of free ADP (Fig. 1), and as ADP_{free} is probably the 'substrate' of the exchange reaction, cf. above and Ref. 32, it is

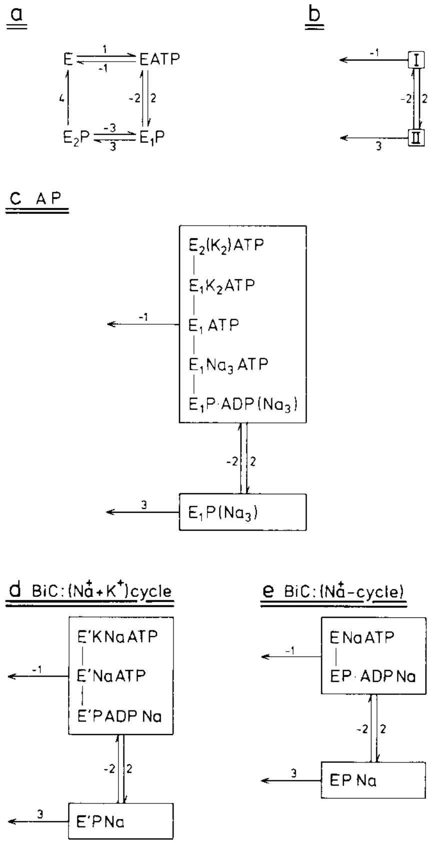

Fig. 4. (a) 4-pool model for the ATP hydrolysis catalyzed by the enzyme, valid for the Albers-Post (AP) as well as for the bicyclic (BiC) mechanism at all conditions, i.e., Na^+-ATPase activity as well as Na^+,K^+-ATPase activity. This model was used for deriving the rate equation for ATP hydrolysis as well as for ADP–ATP exchange in steady-state (see Appendix). The ratio between the rates divided by the concentration of ADP_{free} yielded the constant, K_E, containing only the apparent rate constants k'_{-1}, k'_2, k'_{-2} and k'_3. In (b) is shown the sequence characterized by these four apparent rate constants. I symbolizes the pool of intermediates in equilibrium between addition of substrate and release of the first product. II symbolizes the pool of intermediates in equilibrium between the release of the first product and the conformation change of the phosphorylated enzyme. In (c) is given the content of pools I and II in the AP mechanism according to a recent version (Fig. 1 of Ref. 32). In the AP mechanism this sequence is traversed whether Na^+-ATPase or $(Na^+ + K^+)$-ATPase activity is performed. This is not the case in the BiC mechanism, where (d) represents the sequence at high substrate concentration and K^+ present (the $Na^+ + K^+$ cycle), whereas (e) represent the sequence in the absence of K^+ (the Na^+ cycle). Note that according to the Bic mechanism all of the intermediates of pool I in (d) are different from the intermediates of pool I in (e). The same is true for pools II in (d) and (e). This has been indicated in the figure by adding a prime to the Es in (d). However, the terms E_1 and E_2 were not used in (d) and (e), and it is not in all cases clear how they apply to the intermediates. Nor are the number of sodium ions given — as in (d), they may vary between 1 and 3 [17] and in (e) they may vary between 2 and 3 [16].

tempting to derive a value of 'the ADP_{free} affinity' ($K_{0.5}$) from double-reciprocal plots of the data given in Fig. 1. However, with ADP showing both of the above-mentioned effects, revealing its binding to at least two enzyme intermediates of the reaction cycle, a double reciprocal plot could be only seemingly linear and no meaningful interpretation of the 'ADP_{free} affinity' would be obtainable from it. (Such a figure is shown, in a different context and for different purposes, in the following paper [21]).

However, a characterization of the steps in the sequence

$$\leftarrow E \cdot ATP \rightleftharpoons ADP + EP \rightarrow$$

may be obtained from the ratio between exchange and hydrolysis rates at steady-state, as shown in the Appendix. On the basis of a 4-pool model, valid for the Albert-Post [2,3] as well as for the bicyclic mechanism at all conditions (Fig. 4a), the rate equation was derived for ADP–ATP exchange as well as for ATP hydrolysis in steady-state. The ratio between the rates divided by the concentration of ADP_{free} yielded a constant, K_E, containing only those (apparent) rate constants needed to characterize the enzyme intermediates involved in the sequence

$$\leftarrow E \cdot ATP \rightleftharpoons ADP + EP \rightarrow$$

where $E \cdot ATP$ and EP each symbolize the pool of intermediates in equilibrium in boxes I and II of Fig. 4b, respectively:

$$K_E = \frac{k'_{-2}}{k'_3(1 + k'_2/k'_{-1})} = \frac{v_{exch}}{v_{hydr}} \cdot \frac{1}{[ADP]}$$

where v_{exch} is the ADP–ATP exchange rate and v_{hydr} is the ATP-hydrolysis rate at steady-state, and where the rate constants are apparent rate constants, dependent on the concentrations of those ions that may bind in the appropriate equilibrium pool (see Appendix).

Thus, when the ratio between v_{exch} and v_{hydr} is plotted against the concentration of ADP_{free}, a straight line with intercept zero and slope equal to K_E should be obtained. A replot of the data from Figs. 1 and 2 in terms of ratio versus ADP is given in Fig. 5. Straight lines with intercept zero were obtained at all concentrations of ATP and in the presence as well as in the absence of K^+ (20 mM).

K_E was determined at varying concentrations of K^+ (Fig. 6) and of Na^+ (Fig. 7). The variation of K_E with these ligands was compared to the variation predicted from the two versions of the sequence $EATP \rightleftharpoons EP + ADP \rightarrow$, the Albers-Post version (Fig. 4c) (isolated from Fig. 1 of Ref. 32) and the bicyclic version (Fig. 4d and e) (in the presence and in the absence of K^+, respectively).

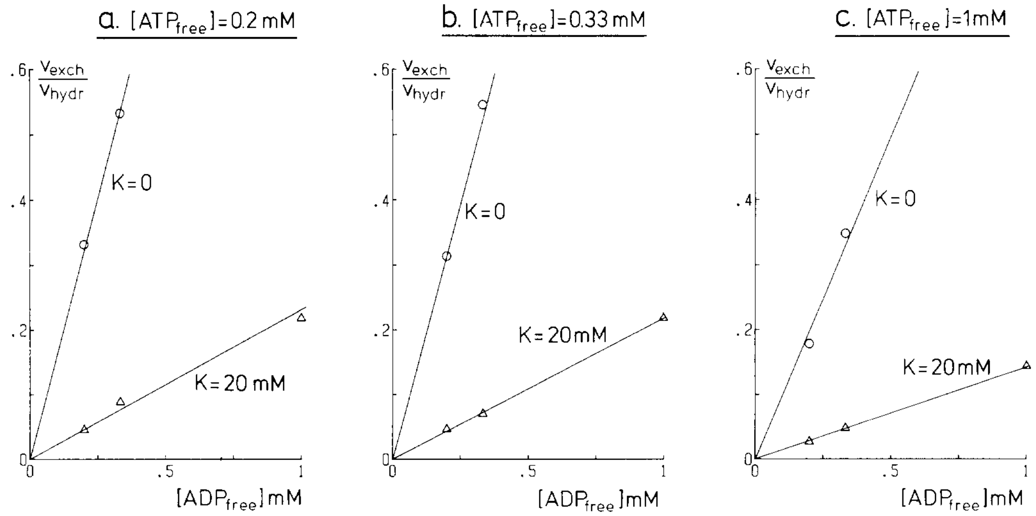

Fig. 5. Ratio between exchange rate (data from Fig. 1) and hydrolysis rate (data from Fig. 2) versus the concentration of ADP_{free} at the three different concentrations of ATP_{free} given in the figure and in the presence as well as in the absence of 20 mM K^+, as indicated. In the absence of K^+, the points at $[ADP_{free}] > 0.33$ mM fall on the extension of the lines.

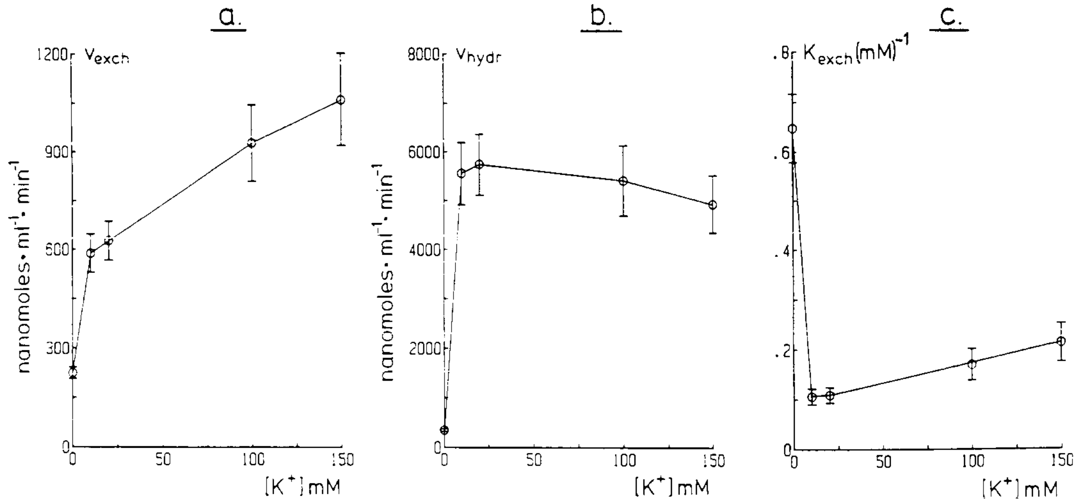

Fig. 6. (a) ADP/ATP exchange rate. (b) ATP hydrolysis rate and (c) K_E as a function of the concentration of K^+. The assay mixture contained 150 mM Na^+, 0.1 mM Mg_{free}, 1.18 mM MgATP and $ATP_{free} = ADP_{free} = 1$ mM in a 30 mM histidine buffer (pH 7.4) at 37 ° C. The enzyme was diluted 70-times before assay in all runs (see legend to Fig. 1). In (a) the specific activity of $[^3H]ADP$ was $1.6 \cdot 10^4$–$3 \cdot 10^4$ cpm/nmol and in (b) the specific activity of $[^{32}P]ATP$ was $2 \cdot 10^3$–$5 \cdot 10^3$ cpm/nmol. The values are the means of three experiments. The lengths of the vertical bars are twice the standard deviation.

It should be emphasized here that no attempt will be made to achieve a complete quantitation of the results. Our more limited objective is to investigate whether our results are compatible with the basic tenet of the Albert-Post scheme: Identity of the phosphoenzyme forms irrespective of the presence of K^+. If not, then the mechanism must be bicyclic. The procedure will therefore be to make predictions regarding the variation of K_E with K^+ and Na^+ for each of the two schemes and compare them with the experimental results.

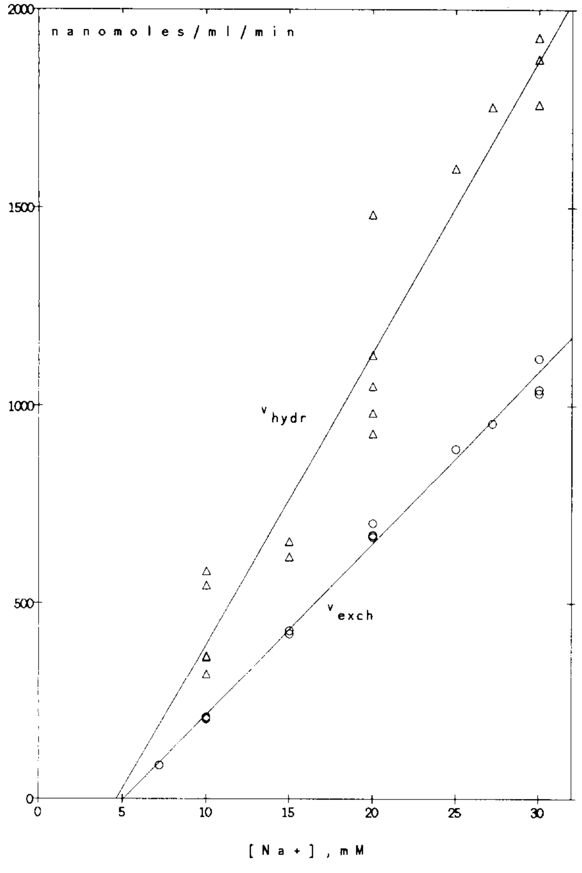

Fig. 7. ADP–ATP exchange rate and ATP hydrolysis rate as a function of the concentration of Na^+. The assay mixture contained 150 mM K^+, 0.1 mM Mg_{free}, 1.18 mM MgATP and $ATP_{free} = ADP_{free} = 1$ mM in a 30 mM histidine buffer (pH 7.4) at 37 ° C. The enzyme dilution and the specific activities of $[^3H]ADP$ and $[^{32}P]ATP$ were the same as in Fig. 6. The rates are individual measurements (means of double determinations) from three different experiments. The lines are the least-squares regression lines:

$$V_{exch} = (43.6 \pm 0.8)\,[Na^+] - (220 \pm 16)$$

abscissa intercept 5.05 ± 0.38

$$V_{hydr} = (73.7 \pm 4.9)\,[Na^+] - (341 \pm 99)$$

abscissa intercept 4.6 ± 1.37

K_E as a function of K^+ at constant $Na^+ = 150$ mM

In none of the sequences (Albert-Post, Fig. 4c, and bicyclic, Fig. 4d) does K^+ bind in pool II, i.e., k'_{-2} and k'_3 are independent of K^+ in both schemes. Also, in both schemes k'_2 becomes a decreasing function of K^+, as K^+ binds in pool I to species that do not split into ADP and EP. Finally, in both schemes k'_{-1} should increase with increasing K^+, as a consequence of the influence of K^+ on the dissociation constant of the enzyme-ATP complex [33,34]. Thus, the increase in K_E observed when K^+ is increased between 10 and 150 mM (Fig. 6c) is predicted by both schemes, which therefore cannot be distinguished on this basis.

However, when the K^+ concentration is decreased, the Albert-Post sequence in Fig. 4c predicts a smooth decrease in K_E, extrapolating to a value at $K^+ = 0$ characterized by the k'_2 and k'_{-1} obtained when no more K^+ is bound in pool I. This is clearly not in accordance with the experimental results (Fig. 6c).

In the bicyclic model the basic idea is that the enzyme intermediates formed at high substrate concentrations in the presence of K^+ (Fig. 4d) are different from those formed in its absence (Fig. 4e). When the intermediates are different (symbolized by the prime on the E in Fig. 4d and no prime on the E in Fig. 4e) the corresponding rate constants are different too, and therefore the bicyclic mechanism predicts a value of K_E at $K^+ = 0$ that is entirely different from the extrapolated value obtained from the function of K_E versus K^+ at $K^+ = 0$. With a bicyclic reaction mechanism, the experimental value of K_E will depend on whether one or the other cycle predominates, but no a priori prediction of the relative values obtained in the two cases can be made, since they depend on entirely different sets of rate constants. The fact that the value of K_E in the absence of K^+ is some 6-times larger than the value obtained in the presence of K^+ supports the notion that different rate constants, and therefore different kinetic cycles, as in the bicyclic model, but contrary to the Albert-Post scheme, are involved in the two situations.

A stimulating effect of K^+ on the exchange rate in the presence of high concentrations of Na^+ and nucleotides (Fig. 6a) has been observed several times [35–38], although the opposite effect has also been reported [39].

K_E as a function of Na^+ at constant $K^+ = 150$ mM

As stated in the Introduction (point 2) our previous steady-state kinetic investigations [14,15, 17] seemed to show that Na^+ and K^+ are most probably bound simultaneously to the intermediate that binds substrate at physiological conditions, i.e., at millimolar concentrations of ATP and in the presence of Na^+ and K^+. As may be deduced from Fig. 4d, this feature leads to the prediction that Na^+ does not influence K_E, as Na^+ is present on all the enzyme intermediates involved in the sequence (see Appendix for details).

In the Albers-Post model (Fig. 4c) there is no simultaneous occupancy on the enzyme of the two ions in pool I. In the forward direction, K^+ has to leave (a supposedly low-affinity inner site) before Na^+ binds (to supposedly high-affinity inner site) and phosphorylation becomes possible. In the backward direction Na^+ has to leave (the high-affinity inner site) for K^+ to accelerate (at a low-affinity inner site) the dissociation of ATP. In other words, at high K^+ concentrations relatively small concentrations of Na^+ (because of the high-affinity Na^+ site involved) should increase k'_2 and decrease k'_{-1}, resulting in a decrease in K_E if this parameter is measured as a function of Na^+ (see Appendix).

The experimental results given in Fig. 7 on the influence of Na^+ on the exchange rate (a) and the hydrolysis rate (b) at $K^+ = 150$ mM is in accord only with the scheme in Fig. 4d: The exchange rate as well as the hydrolysis rate, although both sigmoidal at very low concentrations of Na^+, become linear functions of the Na^+ concentration, and both intersect the abscissa in the same point. It follows that K_E is independent of the Na^+ concentration within the concentration range shown, thus supporting the simultaneous presence of Na^+ and K^+ on the enzyme in pool I.

This is most easily seen as follows: If, in the limited Na^+ concentration range considered, we have

$$v_{\text{hydr}} = a_h + b_h \cdot [Na^+] \tag{1}$$

$$v_{\text{exch}} = a_e + b_e \cdot [Na^+] \tag{2}$$

and, in addition, the two lines intersect the abscissa in the same point, then

$$\frac{b_h}{a_h} = \frac{b_e}{a_e} \tag{3}$$

and thus

$$\frac{v_{hydr}}{v_{exch}} = \frac{a_h\left(1 + \frac{b_h}{a_h}\cdot[Na^+]\right)}{a_e\left(1 + \frac{b_e}{a_e}\cdot[Na^+]\right)} = \frac{a_h}{a_e} \tag{4}$$

which is a constant independent of $[Na^+]$.

At higher concentrations of Na^+ the exchange rate and the hydrolysis rate both deviate from the straight lines given in Fig. 7. The hydrolysis rate still increases at increasing Na^+ concentrations, but the exchange rate shows a slight decrease at very high Na^+-concentrations. This is apparently a low-affinity effect of Na^+, probably relating to outward-facing sites, but it has no bearing on the argument above, which is concerned only with the effect of relatively small Na^+ concentrations relevant for a supposedly high-affinity inner side Na^+ site.

The influence of Na^+ on the ADP–ATP exchange rate under different conditions has been the subject of many investigations (see, e.g., Refs. 36, 38, 40, 41), but to our knowledge the Na^+ influence has not been studied in the presence of a high concentration of K^+.

Recently, Beaugé and Campos [43] published their work on ADP–ATP exchange, also studying simultaneously the hydrolysis and the exchange reaction (but without exploiting their ratio). We defer a discussion of their results to the subsequent paper [21], dealing with a similar study of the exchange, but at micromolar ATP concentrations.

It should be noted that the brain enzyme preparation apparently consists of approximately equal amounts of two isoenzymes (see Materials and Methods). The extent to which this heterogeneity may influence the kinetics is at present under investigation [44].

It should be noticed also that the conclusions reached above rest on the assumption that the phosphorylated intermediates formed in the absence of K^+, E_1P and E_2P, are not in rapid equilibrium. A detailed discussion of this assumption is found in the following paper [21].

In summary, the Albers-Post mechanism was found insufficient to account for the reported results: At high ATP concentrations in the presence of K^+, the intermediate from which ADP is released as a product, or the intermediate to which it may add in the backward direction, or both, are different from the corresponding intermediates obtained when K^+ is absent. Also, the results are consistent with the simultaneous presence of Na^+ and K^+ on the enzyme in part of the reaction cycle. Finally, while ligand effects on the exchange rate may be difficult to interpret, due to the possible interaction of the ligand with other intermediates of the mechanism, the ratio v_{exch}/v_{hydr} was shown to be useful in singling out the ligand effect on the partial reaction involving the isotope exchange.

Appendix

We present here the analysis forming the basis for the conclusions in the main text.

We consider the model in Fig. 4a. This schematic model describes the kinetics of Na^+-ATPase (absence of K^+) as well as of $(Na^+ + K^+)$-ATPase (millimolar substrate and the presence of both Na^+ and K^+), though the interpretation of the intermediates is different in the Albers-Post scheme and the bicyclic scheme (see text).

The isotope exchange for ADP to ATP proceeds through the steps

$$E_1P + ADP^* \rightarrow EATP^* \rightarrow E + ATP^*$$

Using Cleland's diagram procedure [42] to derive the steady-state exchange rate, we obtain:

$$v_{exch} = \frac{k_{-1}k_{-2}\cdot[ADP]}{k_{-1} + k_2}[E_1P] \tag{A1}$$

It is known from experiments [9] that the rate constant k_{-3}, leading from E_2P to E_1P, is much smaller than k_3, and thus may be ignored. With this assumption, which is discussed in detail in the following paper [21], we have

$$v_{hydr} = k_3\cdot[E_1P] \tag{A2}$$

and, hence, eliminating $[\text{E}_1\text{P}]$ we obtain

$$v_{\text{exch}} = K_{\text{E}} \cdot [\text{ADP}] \cdot v_{\text{hydr}} \tag{A3}$$

with

$$K_{\text{E}} = \frac{k_{-2}}{k_3(1 + k_2/k_{-1})} \tag{A4}$$

A ligand, L, may influence the kinetics by binding to one or more intermediates in Fig. 4a. Thus, from Eqn. A3, L will activate the exchange if its presence results in an increase in v_{hydr}, an increase in K_{E}, or both, or if it decreases v_{hydr} and increases K_{E} (or vice versa), provided that the increase more than offsets the decrease.

The form of Eqn. A3 suggests a convenient experimental parameter

$$\eta = \frac{v_{\text{exch}}}{v_{\text{hydr}}} = K_{\text{E}} \cdot [\text{ADP}] \tag{A5}$$

A plot of η as a function of the ADP concentration directly yields K_{E} (see Fig. 5), valid for a given set of conditions. For the purposes of this paper, we denote a compound that increases or decreases, K_{E}, irrespective of its effect on the hydrolysis rate, v_{hydr}, a specific activator or inhibitor, respectively, for ADP–ATP exchange.

It is seen that any complicating effect of a compound (ligand, substrate or product) on the kinetics resulting from its interaction with kinetic states other than EATP and E_1P is immaterial for the values of the ratio η, and hence for K_{E}, because K_{E} is defined solely in terms of apparent rate constants leading away from the two kinetic intermediates with which ADP is directly involved in the exchange reaction. Thus, interaction of the compound with these two states only is manifested in K_{E}.

We consider first the case of the two ligands Na^+ and K^+, binding in pools I and II (Fig. 4b) corresponding to the Post-Albers scheme (Fig. 4c), where K^+ has to leave prior to Na^+ binding in the forward direction. For simplicity, we consider only one ion binding in each case. The two pools are then schematically

$$\xleftarrow{-1} \text{EKATP} = \text{EATP} = \text{ENaATP} \underset{2}{\overset{-2}{\rightleftharpoons}} \text{E}_1\text{PNa} \xrightarrow{3}$$

Scheme I.

where the equality signs indicate rapid equilibrium within a pool. If ENaATP and EKATP both are able to release ATP, but with different intrinsic rate constants $k_{-1\text{N}}$ and $k_{-1\text{K}}$, respectively, while only the former may release ADP, the apparent rate constants are

$$k'_{-1} = \frac{k_{-1\text{K}} \cdot K/K_{\text{K}} + k_{-1\text{N}} \cdot Na/K_{\text{Na}}}{1 + K/K_{\text{K}} + Na/K_{\text{Na}}} \tag{A6}$$

$$k'_2 = \frac{k_2 \cdot Na/K_{\text{Na}}}{1 + K/K_{\text{K}} + Na/K_{\text{Na}}} \tag{A7}$$

$$k'_{-2} = k_{-2}; \quad k'_3 = k_3, \tag{A8}$$

where K_{K} and K_{Na} are the K^+ and Na^+ dissociation constants for EKATP and ENaATP, respectively. K_{E} thus becomes

$$K_{\text{E}} = \frac{k'_{-2}}{k'_3(1 + k'_2/k'_{-1})}$$

$$= \frac{k_{-2}}{k_3} \cdot \frac{k_{-1\text{K}} \cdot K/K_{\text{K}} + k_{-1\text{N}} \cdot Na/K_{\text{Na}}}{k_{-1\text{K}} \cdot K/K_{\text{K}} + (k_{-1\text{N}} + k_2) \cdot Na/K_{\text{Na}}} \tag{A9}$$

It is easy to see (by taking derivatives) that, at fixed Na^+, K_{E} is an increasing function of K^+, i.e., K^+ is a specific activator, while at fixed K^+, K_{E} is a decreasing function of Na^+, which thus acts as a specific inhibitor of the exchange. Clearly, K_{E}, as defined by Eqn. A9, cannot increase, as $\text{K} \to 0$, and it is independent of Na^+ only if either the Na^+ terms in Eqn. A9 are completely dominating, or if the K^+ terms dominate. These features are the results of the competition in Scheme I between Na^+ and K^+. On the other hand, if, in pool I, K^+ and Na^+ may bind simultaneously, as in the bicyclic model (Fig. 4d) the scheme is

$$\xleftarrow{-1} \text{EKNaATP} = \text{ENaATP} \underset{2}{\overset{-2}{\rightleftharpoons}} \text{E}_1\text{PNa} \xrightarrow{3}$$

Scheme II.

Instead of Eqns. A6 and A7, we have now

$$k'_{-1} = \frac{k_{-1\text{N}} + k_{-1\text{K}} \cdot K/K_{\text{K}}}{1 + K/K_{\text{K}}} \tag{A10}$$

$$k'_2 = \frac{k_2}{1 + K/K_{\text{K}}} \tag{A11}$$

and, hence, for K_E we obtain

$$K_E = \frac{k_{-2}}{k_3} \cdot \frac{k_{-1N} + k_{-1K} \cdot K/K_K}{k_{-1N} + k_2 + k_{-1K} \cdot K/K_K} \tag{A12}$$

which again is an increasing function of K^+, but is independent of Na^+. The experimental findings that the value of K_E measured at $K^+ = 0$ is about 7-fold larger than the value obtained by extrapolating $K_E(K^+)$ to $K^+ = 0$, and that at $K^+ = 150$ mM K_E is independent of Na^+ in the range 0–30 mM, thus favor the bicyclic scheme, according to which K_E in the absence of K^+ is comprised of rate constants characterizing a reaction sequence which is different from that exhibited at finite K^+ (and high nucleotide concentration), and, in the latter case, Na^+ and K^+ are simultaneously present on the enzyme in part of the reaction cycle.

It should be emphasized that it is immaterial for the argument above whether or not, in Fig. 4c, the step $E_2KATP–E_1KATP$ is included in the 'equilibrium pool' I. It is not difficult to show that if that step is a separate, steady-state, step, the form of K_E, with particular reference to its dependence of K^+, is again

$$K_E = C \cdot \frac{a + bK}{D + bk} \tag{A13}$$

with $D > a$ (a, b, C and D are constants, cf. Eqn. A12), and this is a monotonically increasing function of K^+.

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