Biochimica et Biophysica Acta 937 (1988) 63–72
Elsevier

BBA 73821

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

Liselotte Plesner [a] and Igor W. Plesner [b]

[a] *Institute of Biophysics and* [b] *Department of Chemistry, Physical Chemistry Division, University of Aarhus, Aarhus (Denmark)*

(Received 18 March 1987)
(Revised manuscript received 29 September 1987)

Key words: ATPase, Na^+/K^+-; ADP–ATP exchange; ATP hydrolysis; Steady-state kinetics; Cation binding; (Ox brain)

The ATP hydrolysis rate and the ADP–ATP exchange rate of ($Na^+ + K^+$)-ATPase from ox brain were measured at 10 μM Mg^{2+}_{free} and at micromolar concentrations of free ATP and ADP. (1) In the absence of K^+, substrate inhibition of the hydrolysis rate was observed. It disappeared at low Na^+ and diminished at increasing concentrations of ADP. This was interpreted in terms of free ATP binding to E_1P. In support of this interpretation, free ATP was found to competitively inhibit ADP–ATP exchange. (2) In the presence of K^+, substrate activation of the hydrolysis rate was observed. Increasing (μM) concentrations of ADP did not give rise to competitive inhibition in contrast to the situation in the absence of K^+ (cf. 1, above). This was interpreted to show that at micromolar substrate, some low-affinity, high-turnover $Na^+ + K^+$ activity is possible, provided the Mg^{2+} concentration is low. (3) While small concentrations of K^+ increased the hydrolysis rate (cf. 2) they decreased the rate of ADP–ATP exchange. To elucidate this phenomenon, parallel measurements of exchange and hydrolysis rates were performed over a wide range of ATP and ADP concentrations, with and without K^+. If, in the presence and absence of K^+, ADP (and ATP competing) are binding to the same phosphorylated intermediate for the backward reaction, it places quantitative restrictions on the ratio of rate constants with and without K^+. The results did not conform to these restrictions, and the discrepancy is taken as evidence for the necessity for a bicyclic scheme for the action of the ($Na^+ + K^+$)-ATPase. (4) An earlier statement concerning the nature of the phosphoenzyme obtained in the presence of Na^+ and K^+ is amended.

Introduction

The present paper is an extension of the investigation begun in the preceding article [1] on the ouabain-sensitive $Na^+ + K^+$-activated ATPase (EC 3.6.1.37). The purpose of the work to be reported below was to compare the kinetic properties of the ADP-sensitive phosphorylated intermediated formed in the presence and in the absence of K^+.

Materials and Methods

Details of the enzyme preparation and of the assay of the ATP hydrolysis and ADP–ATP exchange rates are given in the preceding paper [1] and in the legends to the figures. Whenever ADP was present in the assay, it was ensured by dilu-

Correspondence: L. Plesner, Institute of Biophysics, Physical Chemistry Division, University of Aarhus, DK-8000 Aarhus C, Denmark.

tion of the enzyme that the fastest of the two opposing processes (ATP hydrolysis and ADP–ATP exchange) changed by less than 10% the concentration of the species (MgATP or ADP_{free}) that was present in the lowest concentration. For the calculation of the enzyme dilution it was necessary (at high rates of ADP–ATP exchange) to take into account that [^{3}H]-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. The ouabain-insensitive activity was measured as a blank corresponding to each assay tube containing 1 mM of ouabain.

Results and Discussion

Substrate inhibition in the absence of K^+

We first consider the substrate curve at micromolar substrate in the absence of ADP and K^+ and at $[Mg^{2+}_{free}] = 10$ mM, shown in double reciprocal form in Fig. 1, upper curve.

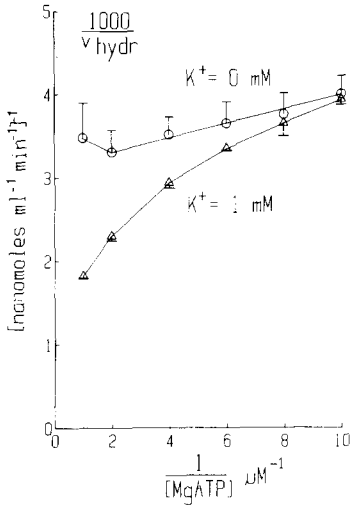

Fig. 1. The reciprocal of the ATP hydrolysis rate at steady-state versus the reciprocal of the substrate (MgATP) concentration. The assay mixture contained 150 mM Na^+ and 0.01 mM Mg^{2+}_{free} in a 30 mM histidine buffer (pH 7.4) at 37 °C. The rate is that corresponding to undiluted enzyme, containing 3.21 mg protein per ml. The enzyme was diluted 10-times in the assay and 4000-times before assay. The specific activity of ATP was $4 \cdot 10^5$ cpm/nmol. The concentration of ATP was 0.85–8.5 μM, i.e., 8.5 times the MgATP concentration. A ouabain blank containing 1 mM of ouabain was measured and subtracted corresponding to each assay tube. The assay time and the temperature were 10 min and 37 °C, respectively. The values are means of three determinations with $K^+ = 1$ mM (△) and six determinations without K^+ (○). The lengths of the vertical bars are the standard deviation. The lines are drawn by eye.

Substrate inhibition is observed, and although substrate inhibition has been reported at higher concentrations of MgATP [1,2], the finding was somewhat unexpected at 1 μM MgATP. However, as discussed in the preceding paper [1], the first product of the reaction is probably free ADP [3–5], and therefore it seems reasonable to hypothesize that free ATP, acting as a product analog, can inhibit the hydrolysis by binding to E_1P, thereby slowing down its turnover. It should be kept in mind that with the free Mg^{2+} concentration used in these experiments, because $K_{d\ MgATP} = 85$ μM [6] we always have $[ATP_{free}] = 8.5 \cdot [MgATP]$.

This hypothesis entails several predictions. Thus, under conditions with low steady-state concentration of E_1P, the ATP inhibition should be decreased or disappear. An experiment to test this prediction is shown in Fig. 2. Low Na^+ concentrations are known to decrease the steady-state concentration of E_1P [2,7–9], and, as seen in Fig. 2, lower curve, the ATP inhibition is not detectable at $[Na^+] = 5$ mM.

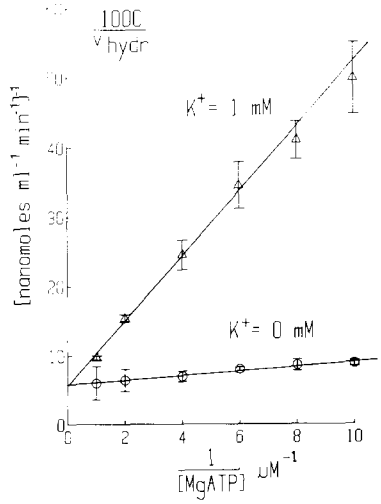

Fig. 2. Double-reciprocal plot of ATP hydrolysis rate versus substrate (MgATP) concentration. All conditions were the same as in Fig. 1, except that the Na^+ concentration was 5 mM. The values are means of four determinations with $K^+ = 1$ mM (△) and three determinations without K^+ (○). The lengths of the vertical bars are twice the standard deviation. The lines are drawn by eye.

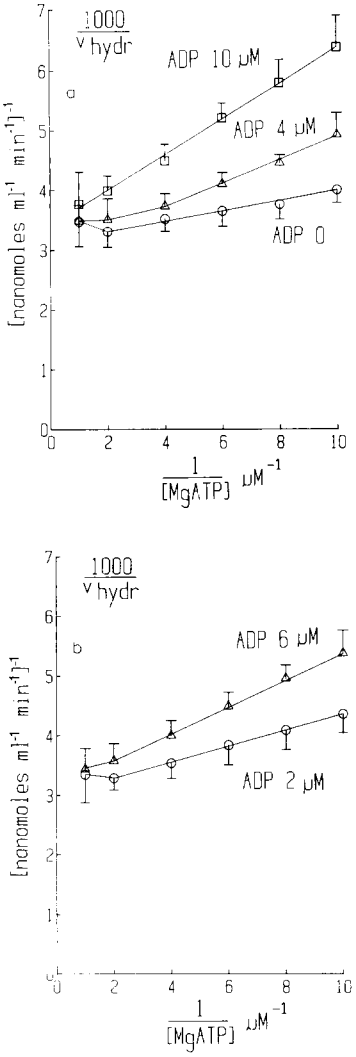

Fig. 3. (a, b) Double-reciprocal plot of ATP hydrolysis rate versus substrate (MgATP) concentration. $Na^+ = 150$ mM and all other conditions were the same as in Fig. 1 upper curve, where $[K^+] = 0$. The ADP concentrations were as indicated in the figure. The values are means of six determinations. The lengths of the vertical bars are the standard deviation. The lines are drawn by eye.

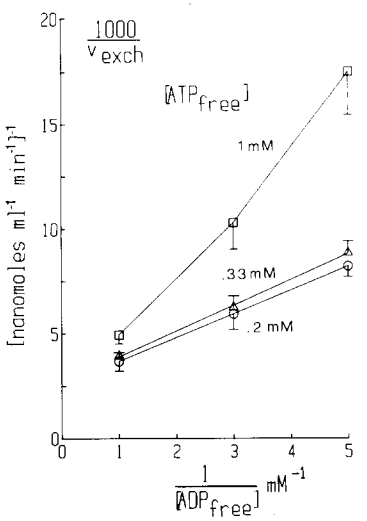

Fig. 4. Double-reciprocal plot of the ADP–ATP exchange rate at steady-state versus the concentration of ADP_{free}. The ADP–ATP exchange rate was measured at the three different concentrations of ATP_{free} given in the figure. The assay mixture contained 150 mM Na^+ and 0.1 mM Mg^{2+}_{free}. The rate is given in nmol of ATP formed per min per ml of undiluted enzyme. The enzyme was diluted 6.4-times in the assay and 100-times before assay. The specific activity of [^{3}H]ADP was $4 \cdot 10^4$ cpm/nmol. The values are means of double determination for three experiments. The lengths of the vertical bars are the standard deviation. The lines are drawn by eye.

If free ATP binds to E_1P, it presumably does so in competition with ADP. To test this prediction, the experiments shown in Fig. 3 were performed. The double-reciprocal plots of the steady-state rate measured at various concentrations of ADP clearly indicate decreasing substrate inhibition as the ADP concentration increases, and at $[ADP_{free}]$ = 10 μM apparent Michaelis-Menten kinetics are obtained.

A third prediction following from the hypothesis of ATP binding to E_1P as an ADP analog is that ATP should show competitive inhibition of ADP–ATP exchange. This effect is demonstrated in Fig. 4.

The inhibitory effects of ATP on v_{hydr} and v_{exch} may be analyzed in more detail as follows. We consider the sequence of steps in Scheme I.

$$\underset{-1'}{\longleftarrow} EATP \underset{-2'}{\overset{2'}{\rightleftarrows}} E_1P \underset{-3'}{\overset{3'}{\rightleftarrows}} E_2P$$

Scheme I.

The hydrolysis rate is obtained as the net rate of flow from E_1P to E_2P

$$v_{hydr} = k'_3[E_1P] - k'_{-3}[E_2P] \quad (1)$$

and the exchange rate is given by (see preceding

paper [1])

$$v_{exch} = \frac{k'_{-2}[ADP]}{1 + k'_2/k'_{-1}}[E_1P] \quad (2)$$

If free ATP binds to E_1P in rapid equilibrium (dissociation constant K_{ATP}) and the adduct can be converted to E_2P but not bind ADP, the apparent rate constants k'_3 and k'_{-2} leading away from E_1P have the form

$$k'_3 = \frac{k_3^o + k_3^a \cdot [ATP]/K_{ATP}}{1 + [ATP]/K_{ATP}} \quad (3)$$

where the superscripts 'a' and 'o' indicate whether or not ATP is bound to the reacting species, and $k_3^a < k_3^o$ to account for inhibition of the hydrolysis, and

$$k'_{-2} = \frac{k_{-2}}{1 + [ATP]/K_{ATP}} \quad (4)$$

[ATP] denotes the concentration of free ATP.

If we assume that the second term in Eqn. 1 is negligible (see below for a discussion of this) we obtain for the two rates

$$v_{hydr} = \frac{k_3^o + k_3^a[ATP]/K_{ATP}}{1 + [ATP]/K_{ATP}} \cdot [E_1P] \quad (5)$$

$$v_{exch} = \frac{k_{-2}[ADP]}{(1 + [ATP]/K_{ATP})(1 + k'_2/k'_{-1})}[E_1P] \quad (6)$$

If $[E_1P]$ is optimal (saturating MgATP) Eqn. 6 shows the pattern of inhibition by free ATP to be expected. It should be noted that sufficiently high (i.e., saturating) concentrations of the substrate MgATP is difficult to attain in the presence of very low free Mg^{2+} concentration. For this reason the experiments shown in Fig. 4 were carried out with 100 μM free Mg^{2+}, in contrast to the 10 μM concentration used in all other experiments in this paper.

We have shown (preceding paper) that a valuable parameter for kinetic characterization of the species E_1P and EATP in Scheme I is obtained from the ratio v_{exch}/v_{hydr}:

$$\frac{v_{exch}}{v_{hydr}} = K_E \cdot [ADP] = \frac{k_{-2}[ADP]}{(k_3^o + k_3^a[ATP]/K_{ATP})(1 + k'_2/k'_{-1})} \quad (7)$$

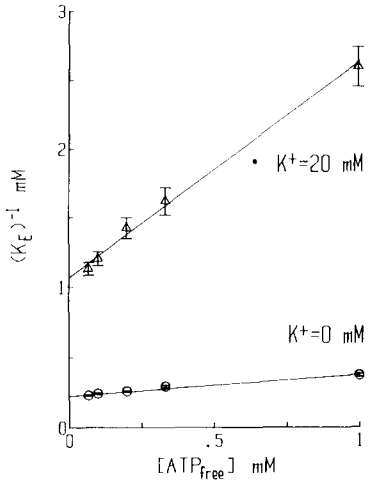

Fig. 5. K_E^{-1} (for definition, see text) versus [ATP_{free}] at 150 mM Na^+ and 0.01 mM Mg^{2+}_{free}, and at 0 and 20 mM K^+ as indicated. The values are means of 3–4 determinations. The lines are the least-squares regression lines, and the lengths of the vertical bars are twice the standard deviation.

where Eqns. 5 and 6 have been used. This shows that $1/K_E$ should be a linear function of [ATP]:

$$K_E^{-1} = \frac{k_3^o}{k_{-2}}(1 + k'_2/k'_{-1}) + \frac{k_3^a}{k_{-2}}(1 + k'_2/k'_{-1})\frac{[ATP]}{K_{ATP}} \quad (8)$$

The experimental results for $1/K_E$ derived from the data are shown as a function of [ATP] in Fig. 5, lower line. The linear relationship obtained appears to conform to Eqn. (8). Thus, the evidence discussed above is consistent with the Albers-Post model [28,29], if binding of ATP to E_1P, i.e., action of ATP as a 'product analog', is also incorporated.

Evidence for the binding of ATP to the phosphoenzyme [10,11], to E_1P [12] and to potassium-sensitive phosphoenzyme [13] has been reported previously.

Substrate activation in the presence of K^+

We turn next to the situation in the presence of K^+. As seen from Fig. 1, lower curve, we now obtain a pattern consistent with substrate activation (or negative cooperation).

The upward convex shape of the double reciprocal plot in the presence of K^+ was first reported by Neufeld and Levy [14] and has since been discussed repeatedly (see, e.g., Refs. 15–17).

At higher Mg^{2+} concentrations than the ones used here, K^+ is found to inhibit ATP hydrolysis at very low substrate concentrations [18,19] because of the slowness of the conversion of $E_2(K)$ to E_1K, and at higher Mg^{2+} concentrations only high (millimolar) ATP concentrations are able to bind to $E_2(K)$ and speed up the deocclusion of K^+, causing substrate activation (or negative cooperativity).

The reason why the substrate activation is seen at the low substrate concentrations in these experiments (Fig. 1) is probably the very low Mg^{2+} concentration. We have shown previously [6] that Mg^{2+} is a competitive inhibitor of substrate addition in the presence of K^+. In the absence of K^+, Mg^{2+} causes no, or only a small, reduction in the affinity of ATP binding [6,20–22]; however, the reduction in nucleotide binding affinity caused by K^+ is greatly reduced, when the Mg^{2+} concentration is kept low [23,24].

Activation by K^+ at micromolar concentrations of substrate has been found by others [25–27]. The activation has been attributed to the displacement of Na^+ ions from extracellular sites at which they inhibit [27]. This explanation was based on experiments with resealed red cell ghosts showing the activation to be brought about by extracellular K^+ and to be more marked at a low than at a high concentration of Na^+ in the extracellular medium. In contrast, as seen in Fig. 2, the activation by K^+ in our hands is abolished at low (5 mM) Na^+.

If, as suggested above, the activation caused by K^+ is caused by a part of the hydrolysis passing through the sequence E_2P-E_2PK-E_2K-E_2KATP-E_1KATP-E_1ATP, then this activation of the 'low-affinity' substrate binding should result in a decreased affinity also for ADP competing at the substrate site. It, therefore, supports the explanation proposed here that low concentrations of ADP do not exhibit visible inhibition of the hydrolysis in the presence of K^+ (Fig. 6).

According to the Albers-Post scheme [28,29] the sequence (Scheme I) above has to be traversed also when K^+ is present in the reaction medium. To investigate the possible influence of K^+, it is helpful to consider an extended version of this scheme (Scheme II), in which the ionic interactions have been explicitly, albeit schematically, indicated:

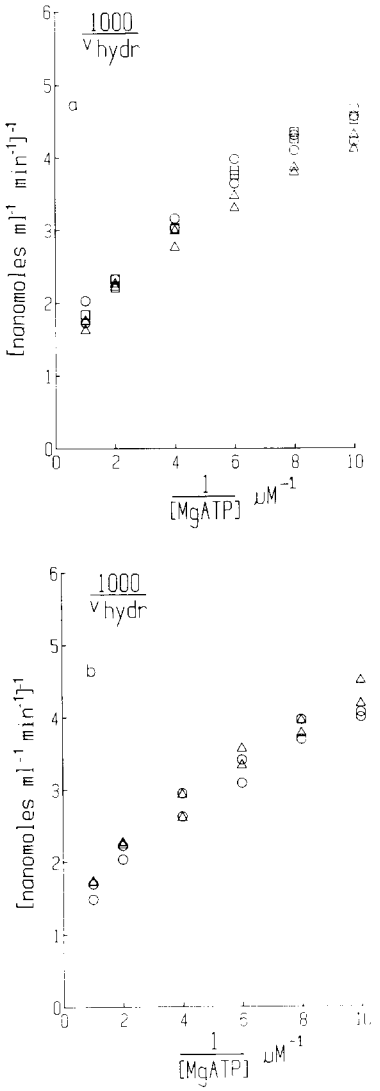

Fig. 6. (a, b) Double-reciprocal plot of ATP hydrolysis rate versus substrate (MgATP) concentration. $[K^+] = 2$ mM. Apart from the K^+ concentration, all conditions were the same as in Fig. 3. (a) $ADP_{free} = 0$ (○), $ADP_{free} = 4$ (△), $ADP_{free} = 10$ μM (□). (b) $ADP_{free} = 2$ μM (○), $ADP_{free} = 6$ μM (△).

$$\xleftarrow{k'_{-1}} \{E_2K_2ATP{=}E_1ATP{=}E_1Na_3ATP\} \underset{k'_{-2}}{\overset{k'_2}{\rightleftarrows}} E_1PNa_3 \underset{k'_{-3}}{\overset{k'_3}{\rightleftarrows}} \{E_2P{=}E_2PK\} \xrightarrow{k'_4} \qquad \text{Scheme II.}$$

(I) (II) (III)

In this scheme an equality sign indicates that rapid equilibrium between the species obtain. Thus, the first three and the last two intermediates, respectively, constitute pools, each of which is to be considered a single kinetic state. ATP may dissociate from each of the species in pool I, but the intrinsic rate constant for ATP dissociation is larger from the K^+-bound species [31,32,35]. The effect of K^+ is, therefore, to increase k'_{-1}, decrease k'_2, and hence decrease the ratio k'_2/k'_{-1}, whereas k'_3 and k'_{-2} are independent of K^+, since K^+ does not bind to pool II.

With this in mind, we consider the effect of K^+, at low substrate concentration (0.353 μM MgATP), on the hydrolysis rate (Fig. 7) and on the exchange rate (Fig. 8). From Eqn. 1 above, the increased hydrolysis rate observed at increased K^+ concentration may be explained by either one of three possibilities: (i) an increase in $[E_1P]$, (ii) a decrease in $[E_2P]$, or (iii) both. From the considerations in the last paragraph it follows that since addition of K^+ leads to a decrease in the ratio k'_2/k'_{-1}, the coefficient of $[E_1P]$ in Eqn. 2 should increase. Therefore, if addition of K^+ leads to an increase, or no change, in $[E_1P]$, the exchange rate should increase, in contrast to the experimental observations in Fig. 8. If the Albers-Post scheme is to explain the results, it follows that under these conditions both $[E_1P]$ and $E_2P]$ in Eqn. 1 must decrease. But this in turn implies that the rate constant k'_{-3} in the absence of K^+ must have a considerable magnitude, such that the reverse reaction rate, from E_2P to E_1P, is almost equal to the forward rate. We return to this point below.

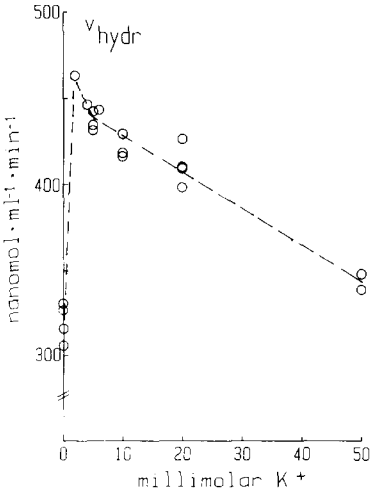

Fig. 7. ATP hydrolysis rate versus $[K^+]$ at 150 mM Na^+, 0.01 mM Mg^{2+}_{free}, 3 μM ATP_{free}, 6 μM ADP_{free}, 0.353 μM MgATP. Buffer, blank, enzyme dilution and assay conditions are described in the legend to Fig. 1. The line is drawn by eye.

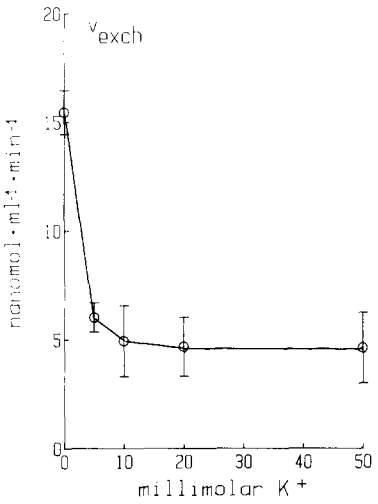

Fig. 8. ADP–ATP exchange rate at steady-state versus the concentration of K^+. All concentrations and conditions were identical to those given in the legend to Fig. 7. The specific activity of $[^3H]$ADP was $6 \cdot 10^6$ cpm/nmol. The values are means of three or four determinations, and the lengths of the vertical bars are twice the standard deviation. The line is drawn by eye.

It is instructive to use the parameter K_E in the presence of K^+ as we did above in its absence. At 20 mM K^+ we would expect that the E_2P concentration is virtually zero, and hence we again obtain Eqn. 8 for the reciprocal of K_E as a function of (free) ATP. According to the model (Scheme II), only the ratio k'_2/k'_{-1} is affected by K^+, and the magnitude of the ratio should be lower than in the absence of K^+. Hence, a plot of $1/K_E$ vs. [ATP] should have a smaller slope and intercept, by the same factor, than the previously obtained values in Fig. 5, lower line.

The data with 20 mM K^+ are plotted in Fig. 5, upper line. Again, a linear relationship is obtained, but both slope and intercept are increased, by factors 9 and 5, respectively, in striking contrast to the expectations based on the Albers-Post model.

The phosphorylated intermediates in the presence of K^+

There are three possibilities for explaining these data:

(1) The assumption made above that the backwards rate, $k'_3 \cdot [E_2P]$, in the absence of K^+ can be ignored does not hold.

(2) K^+ binds to the species in the E_1P pool such that k'_3 is increased and k'_{-2} is decreased, and the resulting increase in the ratio k'_3/k'_{-2} more than compensates for the decrease in the ratio k'_2/k'_{-1}.

(3) The phosphorylated intermediates formed in the presence of K^+ are different from those formed in its absence, and hence the rate constants in Eqn. 8 are different in the two situations.

(ad 1) If the back reaction $E_2P \rightarrow E_1P$ cannot be ignored then it may be shown that the parameter $1/K_E$, in terms of the apparent (primed) rate constants in Scheme II, will be given by

$$K_E^{-1} = \frac{k'_3(1 + k'_2/k'_{-1})}{k'_{-2}(1 + k'_{-3}/k'_4)} \tag{9}$$

where we omit the specific influence of ATP on k'_{-2} and k'_3 for clarity. The rate constant k'_4 in the absence of K^+ is much lower than in its presence, whereas the reverse is true for k'_{-3}. But, as discussed above, the ratio k'_2/k'_{-1} also decreases when adding K^+. To account for the observed increase in $1/K_E$ by a factor of about 5 it is necessary that the decrease in the ratio k'_{-3}/k'_4 in the denominator more than compensates for the corresponding decrease in k'_2/k'_{-1}. For example, if addition of 20 mM K^+ decreases k'_2/k'_{-1} from 2 to 1, and if the ratio k'_{-3}/k'_4 in the absence of K^+ is 10, it should decrease, with 20 mM K^+, to 0.5 to correspond to the observed values. But for the enzyme to be reasonably efficient we expect $k'_3 > k'_{-3}$, and hence, when the enzyme is phosphorylated in the absence of K^+, there should be rapid equilibrium between E_1P and E_2P at steady state of the turnover. But when ADP or K^+ is added to such a system, all the phosphoenzyme should disappear as rapidly as when both are added together. This does not seem to be the case (see, e.g., Refs. 2, 37).

(ad 2) Since (free) ATP also appears to bind to E_1P there are two cases to consider: (i) the binding of ATP and K^+ are mutually exclusive, and (ii) they are not. In the former case, the E_1P pool (written here without Na) consists of the species E_1P, E_1PK, and E_1PATP. It may be shown that in this case $1/K_E$ has the form

$$K_E^{-1} = (1 + k'_2/k'_{-1})\left[\frac{k_3^o + k_3^K \cdot [K]/K_K}{k_{-2}^o + k_{-2}^K \cdot [K]/K_K} + \frac{k_3^a}{k_{-2}^o + k_{-2}^K [K]/K_K} \cdot \frac{[ATP]}{K_{ATP}}\right] \tag{10}$$

where the superscripts indicate the ligands bound to E_1P (ATP or K^+), and K_K is the dissociation constant for K^+ from E_1PK. If $k_3^K > k_3^o$, the intercept of $1/K_E$ vs. [ATP] will increase, whereas the slope will decrease when K^+ is added, in contrast to what is observed (Fig. 5).

If, on the other hand, the species $E_1P \cdot ATP \cdot K$ is also possible and can be converted to an E_2P species, we obtain

$$K_E^{-1} = (1 + k'_2/k'_{-1})\left[\frac{k_3^o + k_3^K [K]/K_K}{k_{-2}^o + k_{-2}^K [K]/K_K} + \frac{k_3^a + k_3^{aK}[K]/K_K}{k_{-2}^o + k_{-2}^K [K]/K_K} \cdot \frac{[ATP]}{K_{ATP}}\right] \tag{11}$$

and thus both slope and intercept may increase with K^+, provided that $k_3^K > k_3^o$ and also $k_3^{aK} > k_3^a$. If these conditions hold then one would expect that K^+, in dephosphorylation experiments, should increase the rate constant governing the disappearance of phosphoenzyme from the slowest, E_1P, pool. However, this is not observed [2].

(ad 3) The addition of K^+ to the enzyme gives rise to the appearance at steady-state of a new species, E_2K, at the expense of E_2P and E_1P. If this species is the substrate-adding enzyme form in an entirely new hydrolysis cycle with different phosphoenzyme forms and with a higher turnover number, as has been proposed [19], it is only necessary that a small fraction of the enzyme, at these low substrate concentrations, is engaged in this new cycle, while the remaining part is using the conventional cycle, in order to explain the increase in hydrolysis rate. At the same time, because the E_1P concentration in this latter cycle is decreased, the observed exchange rate is de-

creased. Thus a qualitative explanation of the observed data is obtained.

From these considerations it appears that a bicyclic model, with two completely distinct hydrolysis cycles, provides the most likely explanation of the data.

Actually, a fourth possibility for explaining the data exists which we merely mention here. It is that K^+ may bind to E_1P, and this complex, rather than E_2K, is the substrate-adding enzyme form in a new hydrolysis cycle. Such a model would be in line with ideas proposed by Skou [33] and Skou and Esmann [34], and it has been exploited by one of us in a recent analysis of a dimer model for $(Na^+ + K^+)$-ATPase [36].

It is interesting to note from Fig. 2 that in the presence of K^+ and low (5 mM) Na^+ concentration the substrate activation seen in Fig. 1 (at 150 mM Na^+) is abolished. If E_2K is the starting point for a new hydrolysis cycle, the lack of Na^+ would not be expected to decrease its steady-state concentration, and hence substrate activation should be expected. The apparent lack of it may indicate that Na^+ is necessary, in addition to ATP, to promote deocclusion of K^+, and that therefore Na^+ and K^+ are present simultaneously on the enzyme in part of the reaction cycle, as other kinetic evidence would seem to indicate [44].

Another possibility for explaining the lack of substrate activation in Fig. 2 is the one mentioned above, that E_1P with K^+, rather than E_2K (or E_2KNa), is the initiating enzyme form in the new, more efficient cycle, and with low Na^+ concentration very little E_1P would be expected at steady-state.

In a recent paper, Beaugé and Campos [30] also performed simultaneous measurements of hydrolysis and exchange, using pig kidney ATPase at pH 7.4 and 37 °C. Their objectives and protocol were different from those of the present work, but some of their results are comparable to ours and warrant discussion here. They argue (Ref. 30, Discussion) that simultaneous activation of hydrolysis and exchange by external K^+ is extremely difficult to explain unless the cycles of ATP hydrolysis give rise to the same intermediates in the presence of Na^+ alone and of $Na^+ + K^+$. This argument seems somewhat overstated. If the addition of K^+ triggers the onset of a hydrolytic cycle with a much higher V_{max} (leading to activation), the exchange reaction using these new intermediates could very well yield a higher rate also.

However, their argument appears to be based on a statement made by us several times in the paper in which the bicyclic model was first proposed [19], namely that the intermediate (denoted E_x) in the new, more efficient, $Na^+ + K^+$ cycle, corresponding to the phosphoenzyme in the Na^+ cycle, was probably not phosphorylated, and a cycle with no phosphoenzyme will, of course, not contribute any ADP–ATP exchange. Alternatively, we stated that E_x, if it were a phosphoenzyme, could not be acid-stable.

The reason for that — somewhat unfortunate — statement was that, from the data known to us at the time [38–43], it appeared that when phosphoenzyme was formed in the presence of Na^+ and K^+ only about 20% or less of the enzyme was measured as phosphoenzyme. However, from our kinetic data, calculations showed that at saturating substrate, and with Na^+ and K^+, about 65% of the enzyme should be in the form E_x at steady state. This apparent discrepancy could then be resolved if E_x was not a phosphoenzyme (or not acid-stable), and therefore could not be precipitated by acid.

For technical reasons it is, however, doubtful whether a reliable estimate can be obtained of the concentration of the acid-stable, phosphorylated intermediate in the presence of K^+ and saturating MgATP. For the brain enzyme under these conditions (37 °C) the turnover number is 150–170 s^{-1}, and the mean time per turnover is thus less than 7 ms. Obviously, the time for mixing the reaction solution with acid plays a role on this time scale, and unless dephosphorylation is stopped exactly as fast as phosphorylation the value obtained for the amount of phosphoenzyme will be too low.

On this basis, we therefore retract our earlier statement and submit that E_x, in analogy with the Na^+ cycle, probably is a (acid-stable) phosphoenzyme, albeit with kinetic properties that are different from those of phosphoenzyme obtained in the absence of K^+.

The rejection by Beaugé and Campos [30] of the idea of two different cycles in the kinetic mechanism is based mainly on similarities in the response of hydrolysis and exchange to the ad-

dition of K^+. But, actually, the two responses are not quite similar, as the data exhibited in their Table II show. Thus, at 3 mM ATP, 12 mM K^+ stimulated hydrolysis by a factor 15.5, but the exchange only by a factor 5.4. At 0.04 mM ATP, the same K^+ concentration led to a 2.4-fold stimulation of hydrolysis, with (almost) unchanged exchange, and at 0.003 mM ATP both activities were inhibited, but by different factors. On the other hand, when these data are analyzed using $K_E = v_{exch}/(v_{hydr} \cdot ADP)$, the results shown in Table I are obtained. Not only is there considerable quantitative agreement when allowance is made for differences in assay conditions (buffer, Mg^{2+}), between our results and those of Beaugé and Campos (compare Fig. 5 and the values of K_E in Table I), but a consistent action of K^+ is seen: 12 mM K^+ lowers K_E by a factor of about 2.7, in qualitative accord with the results in Fig. 6c of the preceding paper and with Fig. 5, above.

These considerations lead us to consider the results of Beaugé and Campos [30] as a reinforcement of ours, and therefore as supporting our conclusion regarding the necessity for having two different hydrolysis cycles, with different (ADP-sensitive) phosphoenzyme intermediates, in the absence and presence of K^+.

We emphasize that, as discussed above, this conclusion is reached on the presumption that E_1P and E_2P are not in rapid equilibrium in the absence of K^+. This, as well as the problems connected with the measurement of phosphoenzyme in the presence of K^+, raises two questions that must be answered by experiments: (i) how fast is the backward reaction (from $E_2P \rightarrow E_1P$) in the absence of K^+, compared to the forward rate?, and (ii) can one obtain, possibly by extrapolation, a reliable estimate of the concentration of phosphorylated enzyme in the presence of K? These problems are presently under investigation.

TABLE I

EFFECT OF K^+ ON K_E, USING DATA FROM TABLE II OF Ref. 30

$[Na^+] = 120$ mM; pH = 7.4 at 37°C. For other experimental details, see Ref. 30.

ATP (mM)	K^+ (mM)	K_E (mM^{-1})	$K_E(0)/K_E(K^+)$
3	0	0.7	2.8
	12	0.25	
0.04	0	4.4	2.7
	12	1.63	
0.003	0	11.1	2.5
	12	4.4	

Acknowledgements

We are grateful to Mrs. Vinni Ravn for her skillful care with the experimental work. Financial support through grants (to L.P.) from the Danish Medical Research Council, the P. Carl Pedersens Foundation, and from Ingeborg and Leo Dannin's Foundation for Scientific Research is gratefully acknowledged. We also thank P.A.G. Fortes and Irena Klodos for helpful discussions, and Ian Glynn for significant criticism of the original manuscript.

References

1 Plesner, L. and Plesner, I.W. (1988) Biochem. Biophys. Acta 937, 51–62.
2 Nørby, J.G., Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725–759.
3 Glynn, I.M. (1985) in The Enzymes of Biological Membranes, 2nd. Edn. (Martonosi, A.N., ed.), Vol. 3, pp. 28–114, Plenum Press, New York.
4 Post, R.L., Toda, G. and Rodgers, F.N. (1975) J. Biol. Chem. 250, 691–701.
5 Richards, D.E. (1987) J. Physiol. 386, 112.
6 Plesner, L. and Plesner, I.W. (1981) Biochim. Biophys. Acta 643, 449–462.
7 Kuriki, Y. and Racker, E. (1976) Biochemistry 15, 4951–4956.
8 Jørgensen, P.L. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 597, 305–317.
9 Hara, Y. and Nakao, M. (1981) J. Biochem. (Tokyo) 90, 923–931.
10 Askari, A. and Huang, W.-H. (1982) Biochem. Biophys. Res. Commun. 104, 1447–1453.
11 Askari, A. and Huang, W.-H. (1984) J. Biol. Chem. 259, 4169–4176.
12 Lee, J.A. (1985) Ph.D. Thesis, University of California, San Diego.
13 Fukushima, Y., Yamada, S. and Nakao, M. (1984) J. Biochem. (Tokyo) 95, 359–369.
14 Neufeld, A.H. and Levy, H.M. (1969) J. Biol. Chem. 244, 6493–6497.
15 Cantley, L.C., Jr., Cantley, L.G. and Josephson, L. (1978) J. Biol. Chem. 253, 7361–7368.

16 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2357–2366.
17 Plesner, I.W. (1983) Current Topics Membr. Transp. 19, 587–590.
18 Czerwinski, A., Gitelman, H.J. and Welt, L.G. (1967) Am. J. Physiol. 213, 786–792.
19 Plesner, I.W., Plesner, L., Nørby, J.G. and Klodos, I. (1981) Biochim. Biophys. Acta 643, 483–494.
20 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176.
21 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De-Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 649, 533–540.
22 Nørby, J.G. (1983) Current Topics Membr. Transp. 19, 281–314.
23 Kaniike, K., Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 298, 901–905.
24 Moczydlowski, E.G. and Fortes, P.A.G. (1981) J. Biol. Chem. 256, 2346–2356.
25 Blostein, R. and Whittington, E.S. (1973) J. Biol. Chem. 248, 1772–1777.
26 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 246–256.
27 Beaugé, L.A., Glynn, I.M. and Richards, D.E. (1979) J. Physiol. 295, 88P.
28 Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727–756.
29 Post, R.L., Kume, L., Tobin, T., Orcutt, H.B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306–326.
30 Beaugé, L. and Campos, M.A. (1986) J. Physiol. 375, 1–25.
31 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116.
32 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234–5240.
33 Skou, J.C. (1985) in The Sodium Pump (Glynn, I.M. and Ellory, C., eds.), pp. 575–588. The Company of Biologists, Cambridge, U.K.
34 Skou, J.C. and Esmann, M. (1983) Biochim. Biophys. Acta 746, 101–113.
35 Plesner, I.W. and Plesner, L. (1981) Biochim. Biophys. Acta 648, 231–246.
36 Plesner, I.W. (1987) Biophys. J. 51, 69–78.
37 Klodos, I. and Nørby, J.G. (1987) Biochim. Biophys. Acta 897, 302–314.
38 Jørgensen, P.L. (1975) Q. Rev. Biophys. 7, 239–274.
39 Mårdh, S. (1975) Biochim. Biophys. Acta 391, 448–463.
40 Mårdh, S. and Zetterquist, Ö. (1974) Biochim. Biophys. Acta 350, 473–483.
41 Han, C.S., Tobin, T., Akera, T. and Brody, T.M. (1976) Biochim. Biophys. Acta 429, 993–1005.
42 Lowe, A.G. and Smart, J.W. (1977) Biochim. Biophys. Acta 481, 695–705.
43 Froehlich, J.F., Albers, R.W., Koval, G.J., Goebel, R. and Berman, M. (1976) J. Biol. Chem. 251, 2186–2188.
44 Plesner, I.W. and Plesner, L. (1985) Biochim. Biophys. Acta 818, 235–250.