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The effect of cytoplasmic K^+ on the activity of the Na^+/K^+-ATPase

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Experiments with the reconstituted ($Na^+ + K^+$)-ATPase show that besides the ATP-dependent cytoplasmic Na^+-K^+ competition for Na^+ activation there is a high affinity inhibitory effect of cytoplasmic K^+. In contrast to the high affinity K^+ inhibition seen with the unsided preparation at a low ATP especially at a low temperature, the high affinity inhibition by cytoplasmic K^+ does not disappear when the ATP concentration an-or the temperature is increased. The high affinity inhibition by cytoplasmic K^+ is also observed with Cs^+, Li^+ or K^+ as the extracellular cation, but the fractional inhibition is much less pronounced than with Na^+ as the extracellular cation. The results suggest that either there are two populations of enzyme, one with the normal ATP dependent cytoplasmic Na^+-K^+ competition, and another which due to the preparative procedure has lost this ATP sensitivity. Or that the normal enzyme has two pathways for the transition from E_2-P to E_1ATP. One on which the enzyme with the translocated ion binds cytoplasmic K^+ with a high affinity but not ATP, and another on which ATP is bound but not K^+. A kinetic model which can accommodate this is suggested.

Introduction

In the presence of Mg^{2+} and ATP Na^+ has a slight activating effect on the isolated unsided membrane bound ($Na^+ + K^+$)-ATPase. A further addition of K^+ has two effects: K^+ activates with a high affinity and this is due to an effect on the extracellular side of the system. In higher concentrations K^+ inhibits not only this activity but also the low activity seen with Na^+ alone. The low affinity inhibitory effect of K^+ is due to a competition for Na^+ activation on the cytoplasmic side of the system.

When the ATP concentration is lowered from the optimal about 3 mM to 1 μM or less the activating effect of Na^+ alone persists, but the further high affinity activation by K^+ disappears and is replaced by a high affinity inhibition down to a certain fraction of the activity with Na^+ alone. This is followed by the low affinity inhibitory effect which is also seen at a higher ATP concentration [1]. At such low ATP concentrations the hydrolytic activity of ($Na^+ + K^+$)-ATPase with $Na^+ + K^+$ is thus lower at all K^+ concentrations than with Na^+ alone. At a given ATP concentration the high affinity K^+ inhibitory effect is enhanced by a decrease in temperature [1].

The high affinity inhibitory effect of K^+ at a low ATP concentration can be explained from a low rate of deocclusion of extracellular K^+ which has become occluded by the dephosphorylation of E_2-P. The rate of this step becomes lower than the rate limiting step in the hydrolysis with Na^+, but no K^+, the dephosphorylation of E_2-P.

Experiments on reconstituted Na/K-ATPase which show that cytoplasmic K^+ has a high as well as a low affinity inhibitory effect on the Na^+-dependent phosphoenzyme level put a question mark to the above given explanation [2]. On the other hand, no high affinity inhibitory effect of cytoplasmic K^+ on hydrolysis in the presence of a high concentration of Na^+ is observed in experiments with either inverted red blood cells [3], or with reconstituted Na/K-ATPase from electric eel [4].

The present experiments are performed in order to see if it is possible to get an answer to the problem. Is the high affinity inhibitory effect of K^+ seen with the unsided preparation at a low ATP concentration due to an extracellular effect of K^+ or to a cytoplasmic effect.

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The effect of cytoplasmic K^+ which is tested on reconstituted Na/K-ATPase has been compared with the effect of K^+ on the unsided preparation. The enzyme source is rectal glands from shark (*Squalus acanthias*). Enzyme from this tissue when reconstituted accomplish an ATP-dependent Na^+/Na^+ exchange which from the point of view of stoichiometry and electrogenecity behaves like a Na^+/K^+ exchange, suggesting that the reaction with extracellular Na^+ follows the same pathway as with extracellular K^+ [5–9]. The effect of cytoplasmic K^+ has also been tested on the physiological Na^+/K^+ exchange, and the exchange reactions with extracellular K^+ replaced by Cs^+ or Li^+, in order to investigate possible *trans*-ions effects. A preliminary report has been presented [10].

Methods

The methods for preparing the membrane bound enzyme, solubilized enzyme, and its reconstitution into liposomes have been described in previous papers [11,12]. For each of the proteoliposome preparations the fraction of the enzyme molecules oriented inside-out (i:o), right side out (r:o), and as non oriented (n:o) is determined. Typically it amounts to 15% inside out, 65% right side out and 20% non oriented.

The non oriented enzyme molecules are inhibited by preincubation of the samples with 1 mM ouabain in the presence of 5 mM Mg^{2+} and 1 mM inorganic phosphate for 20 min at 20°C (pH 7.0). The inside out molecules are activated by addition of ATP in the presence of the proper ligands and 1 mM ouabain. Under these conditions the non oriented and the right side out molecules are inactive.

The Na^+/Na^+ exchange on i:o enzyme is assayed by the hydrolytic activity as previously described [13]. In the presence of extracellular K^+ (inside vesicles) the test time is kept below 15 s in order to avoid internal depletion of K^+. During this period of time the rate of hydrolysis versus time is linear. Control experiments show that identical results are obtained when Na^+-flux into the proteoliposomes are measured instead of ATPase activity.

The specific hydrolytic activity for Na^+/K^+ exchange of the reconstituted enzyme (with the liposomes made permeable by a low concentration of detergent) is 600–900 μmol P_i/mg protein per h with Na^+ 130 mM, K^+ 20 mM, Mg^{2+} 4 mM, ATP 3 mM (pH 7.0), 20°C. For ATP hydrolysis accompanying Na^+/Na^+ exchange with no K^+, 150 mM Na^+, 1 mM Mg^{2+}, 25 μM ATP at pH 7.0, 20°C it amounts to 60–80 μmol P_i/mg protein per h.

Results

Sidedness of K^+ effects

At 0°C and 1 μM ATP the high affinity inhibitory effect of K^+ with the unsided, membrane bound enzyme is very pronounced as shown in the lower curves in Figs. 1A and B. In panel A the Na^+ and K^+ concentration has been varied inversely with $Na^+ + K^+$ kept constant at 110 mM, whereas in panel B the Na^+

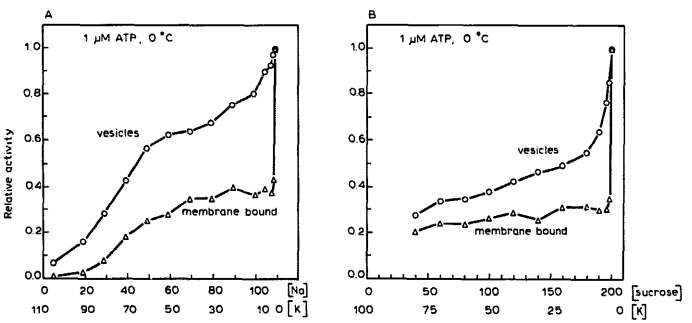


Fig. 1. The $Na^+ + K^+$ activation of unsided, membrane bound Na,K-ATPase (lower curves) and of sided, reconstituted Na,K-ATPase (upper curves). The sided preparation has 115 mM Na^+ and no K^+ on the extracellular side (intraliposomal). (A) $Na^+ + K^+$ in the medium is varied inverse with the sum kept constant at 115 mM. (B) The Na^+ concentration in the medium is constant 30 mM and only K^+ is varied, with isosmolarity ensured by means of sucrose. The temperature is 0°C, 1 μM ATP, 2 mM Mg^{2+}, 30 mM histidine-HCl (pH 7.0). Abscissa: mM Na^+ and K^+ in the extraliposomal medium. Ordinate: hydrolytic activity relative to the activity with 115 mM Na^+ and no K^+.

concentration is kept constant at 30 mM and only K^+ has been varied inversely with sucrose, keeping the osmotic concentration constant. Both the high and the low affinity inhibitory effect of K^+ is seen.

In experiments with the sided preparation (the upper curves labelled vesicles in Figs. 1A and B) there is no K^+ on the extracellular side, the vesicles contain 115 mM Na^+. It is seen that also under these condition, with K^+ exclusively on the cytoplasmic side, is there a high affinity inhibitory effect of K^+. However, the apparent affinity for the high affinity inhibitory effect of cytoplasmic K^+ is lower than with K^+ concomitantly on both extracellular and cytoplasmic sides as with the unsided preparation.

Detergent effects.

The reconstituted enzyme differ from the membrane bound in that it has been dissolved in the non-

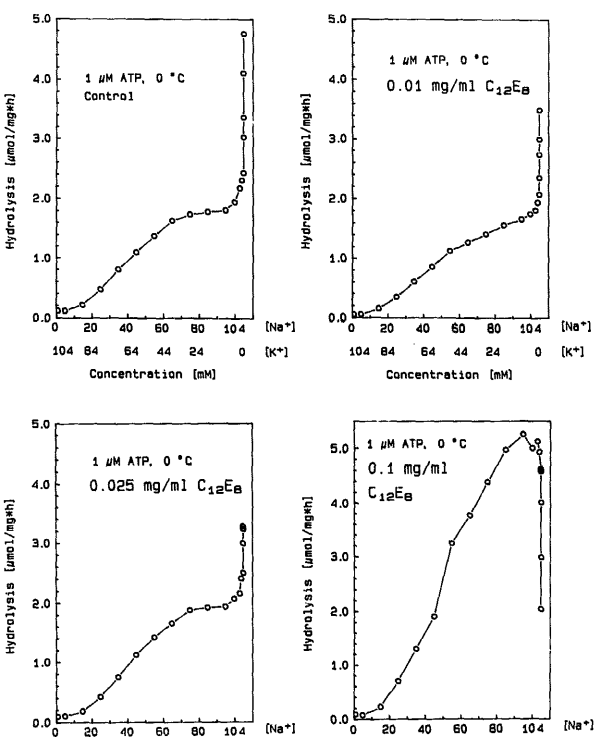
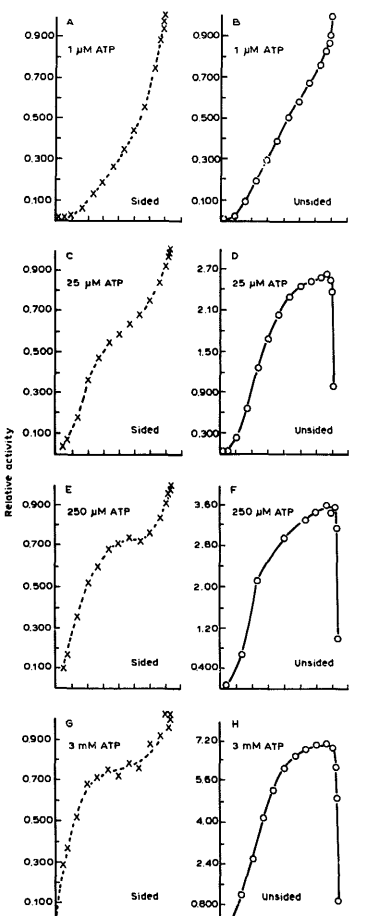


Fig. 2. Effects of the detergent $C_{12}E_8$ on $Na^+ + K^+$ activation of fractured membrane bound Na,K-ATPase. Na^+ and K^+ is varied inverse with the sum kept constant at 115 mM. 1 μM ATP, 2 mM Mg^{2+}, 30 mM histidine-HCl (pH 7.0), 0°C. Abscissa: mM Na^+ and K^+ in the extraliposomal medium. Ordinate: μmol ATP hydrolysed per mg protein per hour. The concentration of $C_{12}E_8$ is given in the figure.



ionic detergent $C_{12}E_8$, and even if the detergent has been removed by adsorption to bio beads, there are still traces retained in the preparation. As it has been shown that the detergent in non solubilizing concentrations has effect on $K_{0.5}$ for ATP and K^+ [14], it is tested if a detergent effect on the high affinity inhibitory effect of K^+ can explain the lower apparent affinity seen with the sided preparation.

In Figs. 2A, B, C and D results are shown in which the experiments in Fig. 1A with the membrane bound unsided preparation have been performed with added increasing amounts of $C_{12}E_8$. The detergent decreases the activity with Na^+ alone. This is due to a decrease in the rate of dephosphorylation of the phosphoenzyme formed in the presence of Na^+ alone (not shown). As the level to which K^+ with the high affinity inhibits is the same without and with 0.01 and 0.025 mg C_{12}/E_8 ml, the decease in activity with Na^+ alone leads to a decrease in the fractional high affinity K^+ inhibition. There is no effect of the detergent on the affinity for the high affinity inhibition by K^+. With a higher $C_{12}E_8$ concentration, 0.1 mg/ml the high affinity inhibitory effect is converted into an activation and with an affinity for K^+ comparable to the affinity for the high affinity inhibition.

Effects of ATP

An effect of the detergent on the reconstituted enzyme cannot explain that the affinity for the high affinity inhibitory effect of K^+ is lower with the reconstituted sided than with the membrane bound unsided enzyme. The difference in affinity may therefore suggest two different effects of K^+. That this is the case is further substantiated from experiments in which the effect of an increase in the ATP concentration is tested, Fig. 3A–H.

In these experiments the effect on the unsided preparation is tested on the fraction of the enzyme which during the reconstitution is incorporated in the membrane in such a way that both sides are exposed, the non oriented enzyme. This ensures that it is enzyme which has been through the same procedure as the reconstituted inside out enzyme and contains the same traces of $C_{12}E_8$. This is performed in order to avoid differences which may be due to an effect of detergent.

Fig. 3. Effect of the ATP concentration on the $Na^+ + K^+$ activation of reconstituted i:o, sided Na,K-ATPase, left panels, and of n:o, unsided Na,K-ATPase, right panels. $Na^+ + K^+$ in the medium is varied inverse with the sum kept constant at 115 mM. The reconstituted i:o enzyme has 115 mM Na^+ extracellular and no K^+. A and B, 1 μM ATP; C and D, 25 μM ATP; E and F, 250 μM ATP; G and H, 3 mM ATP. The Mg^{2+} is in all the experiments 2 mM, 30 mM histidine-HCl (pH 7.0), 23°C. Abscissa: mM Na^+ and K^+. Ordinate: hydrolytic activity relative to the activity with Na^+ alone.

The experiments are performed at room temperature, 23°C. At this temperature the high affinity K^+ inhibition still persist with 1 μM ATP, but the fractional inhibition is lower than at 0°C, compare Figs. 3A and 1A. With an increase in the ATP concentration to 25 μM the high affinity inhibitory effect of K^+ on the unsided preparation disappears and is replaced by the high affinity activation. However, with the sided preparation, which has Na^+ but no K^+ extracellular, an increase in the ATP concentration does not eliminate the high affinity inhibitory effect of cytoplasmic K^+, not even at 3 mM ATP, the optimal for activation in the presence of $Na^+ + K^+$. Similar results are obtained at 0°C (not shown). Increasing the ATP concentration has a pronounced effect on the activation curve at the low Na^+/high K^+ end, whereas only little effect at the high Na^+/low K^+ end, i.e., where the high affinity inhibition by cytoplasmic K^+ is observed.

The high affinity inhibitory effect of K^+ seen with the unsided membrane bound enzyme and the high affinity inhibitory effect of cytoplasmic K^+ seen with the sided preparation thus differ both in affinity and in ATP sensitivity.

With 3 mM ATP the ATP dependent Na^+ activation curve with the sided preparation, the left part of the curve in Fig. 3G, is steeper than the Na^+ activation curve for the unsided preparation, the left part of the curve in Fig. 3H. With the sided preparation which has Na^+ but no K^+ on the extracellular side ATP thus seem to increase the affinity for Na^+ for activation relative to K^+ more than in the experiments with the unsided preparation which has K^+ extracellular.

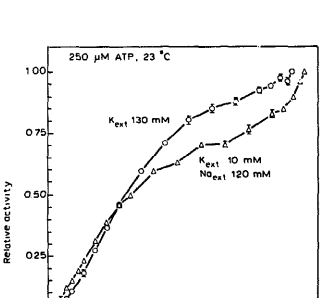


Fig. 4. Activation by cytoplasmic $Na^+ + K^+$ of the hydrolytic activity of reconstituted sided ileo Na,K-ATPase with 130 mM K^+ and with 120 mM Na^+ + 10 mM K^+ extracelluar. $Na^+ + K^+$ on the cytoplasmic side is varied inverse with the sum kept constant at 130 mM. 250 μM ATP, 2 mM Mg^{2+}, 30 mM histidine-HCl (pH 7.0), 23°C. Abscissa: mM Na^+ and K^+. Ordinate: hydrolytic activity in μmol ATP hydrolysed per mg protein per hour.

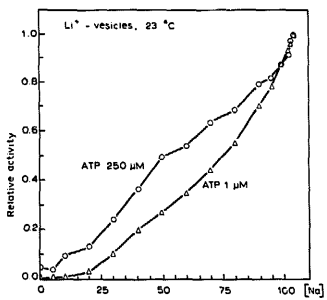


Fig. 5. As in Fig. 4 but with 115 mM Li^+ on the extracellular side in stead of 130 mM K^+ and no Na^+. $Na^+ + K^+$ on the cytoplasmic side 115 mM. 1 μM and 250 μM ATP, respectively, 2 mM Mg^{2+}, 30 mM histidine-HCl (pH 7.0), 23°C. Abscissa: mM Na^+ and K^+. Ordinate: hydrolytic activity relative to the activity with 115 mM Na^+ on the cytoplasmic side.

Trans-effects of extracellular cations

In the experiments shown in Fig. 3 the unsided preparation has K^+ on both sides of the enzyme whereas the sided preparation has no K^+ on the extracellular side. This means that with the unsided preparation it is the activity associated with Na^+/K^+ exchange which is measured, while with the sided it is the activity associated with the Na^+/Na^+ exchange. For methodological reasons it is difficult in the reconstituted system to vary Na^+ and K^+ concomitantly on both extracellular and cytoplasmic sides in order to compare the observed differences of sided and unsided preparations directly. Neither has it been possible to test on the reconstituted system if the high affinity inhibitory effect of K^+ seen with the unsided preparation is due to an extracellular effect of K^+.

In order to test if extracellular K^+ has an effect on the high affinity inhibition by cytoplasmic K^+ the experiments with the sided preparation is repeated with reconstituted enzyme which either has a high K^+-concentration on the extracellular side and no Na^+, or a combination of K^+ and Na^+. In one set of experiments (Fig. 4, upper curve) there is 130 mM K^+ extracellular and no Na^+, in another (Fig. 4, lower curve) 10 mM K^+ + 120 mM Na^+. The test time is below 15 s in order to minimize the effect of the Na^+/K^+ exchange on the intravesicular concentration of K^+. In these experiments the medium K^+ is removed and replaced by

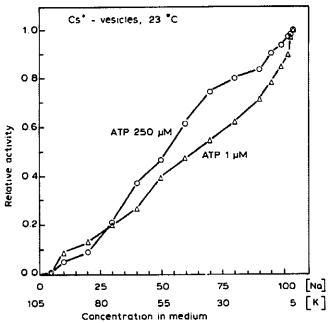


Fig. 6. The experiments in Fig. 5 repeated but with 115 mM Cs^+ extracellular and no Na^+. $Na^+ + K^+$ on the cytoplasmic side 115 mM.

$Tris^+$ by centrifugation of the liposomes through a Sephadex G-50 column.

The results of the experiments with 250 μM ATP at 23°C are shown in Fig. 4. The fraction of the activity which is inhibited by the high affinity effect of cytoplasmic K^+ is substantially higher with 10 mM K^+ + 120 mM Na^+ extracellular than with 130 mM K^+ but no Na^+.

The high affinity inhibition by cytoplasmic K^+ is also observed with 115 mM Li^+ or 115 mM Cs^+ extracellular but no K^+ or Na^+ but with $Na^+ + K^+$ on the cytoplasmic side, Figs. 5 and 6.

A comparison of the high affinity inhibitory effect of cytoplasmic K^+ with different cations extracellular and with 250 μM ATP Figs. 3–6 show that the fractional inhibition by cytoplasmic K^+ is higher when the dominating extracellular cation is Na^+ than in the presence of any of the cations K^+, Li^+, or Cs^+. The absolute value is about 3 times higher with Na^+ than with K^+ but no Na^+ extracellular.

Discussion

The experiments show two different high affinity inhibitory effects of K^+ on the ATPase activity in the presence of Na^+. One is seen with the unsided preparation at a low ATP concentration and low temperature, and this is converted to a high affinity activation when the ATP concentration and/or the temperature is increased. The other is observed during Na^+/Na^+ exchange, or to a minor extent in exchange reactions in which extracellular Na^+ is replaced with K^+, Li^+ or Cs^+, and is due to an effect of cytoplasmic K^+. The high affinity inhibitory effect of cytoplasmic K^+ does not disappear when the ATP concentration and/or the temperature is increased.

The high and the low affinity inhibitory effect of cytoplasmic K^+ on the hydrolysis during Na^+/Na^+ exchange resembles the effects of cytoplasmic K^+ on the level of phosphoenzyme in experiments with reconstituted kidney enzyme in the presence of either extracellular Na^+ or Tris [3]. However, in these experiments the high affinity inhibitory effect of cytoplasmic K^+ decreases when the ATP concentration is increased from 1 μM to 10 μM, or the temperature is decreased in contrast to the effects observed in the present experiments.

As discussed in the introduction the high affinity inhibitory effect of K^+ seen with the unsided preparation can be explained from a rate of deocclusion of K^+, which with the low ATP concentration becomes rate-limiting, and with a rate which is lower than the rate limiting step with Na^+ but no K^+, the dephosphorylation of E_2-P. An increase in the ATP concentration increases the rate of deocclusion of K^+ [16,17] and it becomes higher than the rate of dephosphorylation of E_2-P with Na^+ but no K^+, i.e., the hydrolytic activity with $Na^+ + K^+$ becomes higher than the activity with Na^+ alone.

The high affinity inhibitory effect of cytoplasmic K^+ which is seen also with the higher ATP concentrations in the sided preparation is not seen with the unsided preparation. This does not mean that it does not exist there, but the inhibitory effect of cytoplasmic K^+ is overruled by the activation by extracellular K^+.

But how to explain the high affinity inhibitory effect of cytoplasmic K^+? The experiments suggest that there are two different pathways to E_1Na_3ATP from the E_2 form with the translocated cation. One on which the Na^+ versus K^+ affinity is dependent on the ATP concentration, and another on which it is not. It is not possible to exclude that this is due to two different populations of enzyme, one for which the cytoplasmic Na^+ versus K^+ affinity is ATP dependent, and which is the normal enzyme. And another, which due to the preparative procedure has been changed in such a way, that the ATP effect on the Na^+ versus K^+ affinity on the cytoplasmic side has disappeared. The fraction of the enzyme with the abnormal behaviour will not bee seen in the unsided preparation, since the activating effect of extracellular K^+, as discussed above overrules the inhibitory effect of cytoplasmic K^+. On the other hand, the fractional activity as well as the absolute amount of the activity, which is inhibited by the high affinity effect of cytoplasmic K^+ depends on the cation on the extracellular side. This speaks against the effect being due to two different populations of enzyme.

How to explain the effect if the two pathways are part of the normal reaction? Na^+ which during Na^+/Na^+ exchange is translocated from the outside to

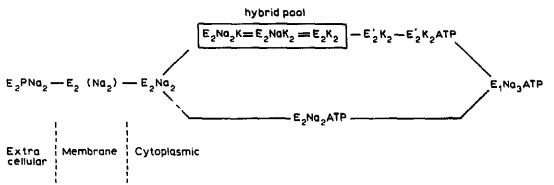


Fig. 7. A model to explain the high affinity inhibitory effect by cytoplasmic K^+ of Na^+/Na^+ exchange. For explanation see text.

the inside, as the occluded $E_2(Na_2)$ has a high rate of spontaneous deocclusion to E_2Na_2. A way to explain the high affinity inhibitory effect of cytoplasmic K^+ is that E_2Na_2 on the one pathway binds cytoplasmic K^+ with a high affinity, i.e., K^+ is bound to the third site, a hybrid form, and in this form the enzyme cannot bind ATP, see Fig. 7. On the other pathway E_2Na_2 binds ATP but not K^+. The E_2Na_2ATP form is converted to E_1Na_3ATP. The hybrid form, E_2Na_2K, exchange Na^+ for K^+, to E_2NaK_2, which releases Na^+ and ends up as E_2K_2. This is a fast reaction which comes to equilibrium, and is followed by a conformational transition to E'_2K_2 which may be an occluded form. This form binds ATP with a low affinity, and with ATP bound it can exchange the two K^+ for two Na^+, and E_2Na_2ATP is converted to E_1Na_3ATP. The high affinity inhibition of cytoplasmic K^+ is due to the accumulation of the enzyme on the hybrid form, the form which has K^+ bound at the third site. In order that the fraction on the hybrid form is ATP independent it is, however, necessary that there is a slow step prior to the binding of ATP. In the model this is assumed to be the step from the hybrid pool to E'_2K_2, but if this form is an occluded form it seems unlikely that this can be the slow step, there must be another step interposed. With such a slow step and choosing proper constants it is possible from the model to mimick the curves which describes the cytoplasmic high affinity inhibitory effect of cytoplasmic K^+ (see Ref. 10).

With K^+ extracellular but no Na^+, K^+ is translocated in stead of Na^+. The $E_2(K_2)$ which is formed, and which has a low rate of spontaneous deocclusion binds ATP with a low affinity. With ATP bound it can exchange K^+ for Na^+ and is converted to E_1Na_3ATP, i.e., there is no or very little of the spontaneous deoccluded E_2K_2 to which cytoplasmic K^+ can bind, and therefore little of the 'hybrid' form with K^+ bound to the third site. Due to this there is little or no high affinity cytoplasmic K^+ inhibition. With 10 mM K^+ + 120 mM Na^+ extracellular there is a high affinity cytoplasmic K^+ inhibition. According to the model this means that with this concentration of $K^+ + Na^+$ extracellular a certain fraction of the turnover is due to translocation of extracellular Na^+.

That the model can mimick the observed curves does of course not mean that this is the explanation. There are so many constants involved of which many are unknown, and no attempt has therefore been made to try to fit the results to the model.

It is not possible from the present experiments to decide whether the explanation of the high affinity inhibition by cytoplasmic K^+ are two fractions of enzyme with different properties or two pathways for the transition from translocation to phosphorylation. For interpretation of kinetic experiments especially at the low ATP concentrations it is important to know the answer. Considering that detergent is used for preparation of the enzyme, it is also for kinetic experiments important to have in mind the pronounced effect of detergents on the effect of Na^+, and of K^+ in the presence of Na^+ at the low ATP concentrations.

Acknowledgements

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