Potassium-induced Changes in Phosphorylation and Dephosphorylation of (Na⁺ + K⁺)-ATPase Observed in the Transient State

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Effects of K⁺ and Na⁺ on the transient state kinetics of (Na⁺ + K⁺)-ATPase from Electrophorus electricus were examined. Exposure of the enzyme to K⁺ for brief intervals prior to the addition of ATP and Na⁺ converts the enzyme to a form (E₂-K) which is transiently less reactive than when all three ligands are added simultaneously. Enzyme is reconverted to the rapidly reacting (E₁) form if Na⁺ is added prior to ATP. Exposure of the ATPase to K⁺ without Na⁺ for 1 to 2 h partially restores the initial phosphate (Pᵢ) burst but greatly depresses the amount of phosphoprotein intermediate (E-P) observed. Experiments in the presence of valinomycin suggest that much of this depression in E-P is due to the presence of sealed vesicles with K⁺ sites sequestered in the interior. Although the results are largely consistent with a simple model in which ATP hydrolysis occurs only through the phosphoenzyme intermediate, the partial restoration of the Pᵢ burst following long term exposure to K⁺ appears to be attributable to a slow change which may allow some ATP hydrolysis to occur without formation of a phosphoenzyme intermediate.

A large body of research on the steady state kinetics of the (Na⁺ + K⁺)-ATPase has suggested that the reaction occurs through phosphorylation and dephosphorylation of the enzyme by a mechanism similar to the following:

\[ E₁ + ATP \rightleftharpoons E₁ \cdot ATP \rightleftharpoons E₁ \cdot P \rightleftharpoons E₁ \cdot P + P \rightleftharpoons E₁ \]  

(1)

The primary role of Na⁺ is postulated to be related to phosphorylation and dephosphorylation of the enzyme by a mechanism similar to the following:

\[ E₁ + ATP \rightleftharpoons E₁ \cdot ATP \rightleftharpoons E₁ \cdot P \rightleftharpoons E₁ \cdot P + P \rightleftharpoons E₁ \]  

(1-3).

Transient state kinetics of this enzyme have also been studied (4-12). When the enzyme is preincubated with Na⁺, either in the presence or absence of K⁺, upon the addition of ATP there is an early burst of phosphate production which is accompanied by a transient overshoot in phosphate (E-P) formation (8, 9, 11). Both the phosphate burst (11) and the E-P overshoot (8) can be suppressed by the addition of K⁺ to the enzyme prior to Na⁺ and ATP. These data are consistent with the proposal by Post et al. (13) that ATPase activity is limited mainly by the rate of conversion of E₁ to E₁, where these are conformations of the enzyme that exist in the absence of ATP, and that the presence of K⁺ in the absence of Na⁺ stabilizes E₂.

The main purpose of this study was to further examine the effects of K⁺ on (Na⁺ + K⁺)-ATPase in the transient state by measuring both E-P formation and phosphate release after incubation of the enzyme in media of varied composition. The results have enabled us to calculate the rates of interconversion of the two nonphosphorylated forms of the ATPase (E₁ and E₂) in the absence of nucleotide. The order of addition of activating ligands is shown to produce changes in the behavior of the enzyme that occur not only in the initial cycle of hydrolysis, but in some cases persist for more than one cycle.

MATERIALS AND METHODS

Microsomal (Na⁺ + K⁺)-ATPase was prepared from electric organ of Electrophorus electricus (14), suspended in distilled H₂O, and stored under liquid N₂.

The apparatus used in the rapid mixing experiments has been described (9, 15). The apparatus contains four syringes which permit the addition of one or two substrates prior to the addition of acid. All experiments were carried out at 21°C. Blanks, which were prepared by manual mixing of the acid denatured enzyme with the appropriate substrates, were subtracted from each sample.

All solutions contained 2.5 mM dithiothreitol, 0.1 mM EDTA, 60 mM Tris-HCl, pH 7.5, and MgCl₂, NaCl, KCl, and ATP, as indicated. Nine per cent perchloric acid containing 1 mM H₂PO₄ was used for quenching the reaction (final concentration, 3% for single mixing, 2.25% for double mixing experiments). [γ-³²P]ATP was prepared using the method of Glynn and Chappell (16). ATP was obtained from Sigma as the Tris or sodium salt, and in some cases from Boehringer as the sodium salt. (No differences were observed in results obtained with ATP from these two sources, even at higher ATP concentrations). All other reagents were analytical grade.

After the reaction had been quenched with perchloric acid, 2-ml samples were assayed for ³²P-labeled protein and ³²P, as described previously (9).

For all curve fitting and simulations we used routines contained in MATLAB, a computer program which has been previously described (17). Curve fitting was done by an iterative least squares method which generates “best fit” values to the experimental data or by visual inspection.

RESULTS

Effects of K⁺ on Phosphoenzyme Formation and P Liberation—Potassium activation of the (Na⁺ + K⁺)-ATPase is believed to take place by activation of the catalytic center for hydrolysis of E₂-P (3). However, there is an optimal concentration above which K⁺ slows the overall ATPase reaction, presumably by inhibiting the conversion of E₂ to E₁ (13). In order to systematically investigate the effects of K⁺ on the
partial reactions of the ATPase, transient phosphoenzyme formation and P_i liberation were measured after mixing ATP and various K^+ concentrations (0 to 20 mM) with enzyme that had been premixed with 100 mM Na^+ (the actual K^+ concentration in these experiments was higher than the added amount by about 0.05 mM, due to the presence of endogenous K^+).

When the enzyme was equilibrated with 100 mM Na^+ and then phosphorylated by the addition of 10 µM [γ-32P]ATP plus 0.5 mM K^+, phosphoenzyme accumulated at a rapid rate and was accompanied by P_i production which increased transiently and then leveled off at a linear rate (Fig. 1). In contrast to the behavior found at 0.5 mM K^+, F_i release in the absence of added K^+ showed only a single phase of hydrolysis following a brief induction period.

When the enzyme was equilibrated with Na^+ and then mixed with ATP and 20 mM K^+, the steady state level of phosphoenzyme decreased while both the initial and final rates of P_i production increased relative to the observations at 0.5 mM K^+. By extrapolating the steady state phase of P_i production to zero reaction time, the burst amplitude was obtained (Table I). The burst size varied with K^+ concentration, with the largest change occurring between 0.5 and 0.75 mM K^+. The increase in burst amplitude as [K^+] was raised was accompanied by a reciprocal decline in the phosphoenzyme level. Saturation of the early burst and maximal reduction of the phosphoenzyme level occurred at 5 mM K^+. Further increase in K^+ had no effect on either of these parameters, but reduced the overall reaction velocity slightly.

**Effects of Order of Addition of Na^+ and K^+**—Equilibration of the enzyme with both Na^+ and K^+ prior to ATP increased the transient phase of phosphate production compared to that seen when K^+ was omitted initially (Fig. 2). A similar stimulation was seen in 10 mM Na^+ and 1 mM K^+. This suggested that K^+ is able to occupy stimulatory sites on the enzyme prior to binding of ATP. Moreover, the addition of K^+ with Na^+ prior to ATP depressed the amount of steady state phosphoenzyme, but did not lead to a proportional change in the steady state rate of P_i production. If most of the phosphate is produced by hydrolysis of the phosphoenzyme, the rate of turnover of this intermediate must be different in the two experiments. Alternatively, part or all of the phosphoenzyme may arise from a side reaction of the enzyme that is sensitive to the sequence of ligand additions.

These hypotheses were tested by examining the transient kinetics of hydrolysis of the phosphoenzyme formed under the experimental conditions of Fig. 2 in a double mixing experiment. The time course of disappearance of phosphoenzyme following addition of excess unlabeled ATP is shown in Fig. 3. In both cases, two exponentials must be used to obtain an adequate fit of the data. Enzyme that was phosphorylated after exposure to Na^+ in the absence of K^+ showed a larger fraction of enzyme that dephosphorylated very slowly. This confirmed that a large fraction of the phosphoenzyme seen after preincubation with Na^+ and without K^+ is relatively inert, and does not contribute substantially to phosphate production. This was reflected not only in different burst sizes but also in slightly different steady state rates of P_i production.

**Properties of the Stable Phosphoenzyme**—The rate at which K^+ is able to depress formation of the slowly decaying

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**Fig. 1. Transient state phosphorylation and phosphate release of (Na^+ + K^+)-ATPase at 0.5 mM KCl.** Enzyme syringe contained electroplax microsomes (1.2 mg of protein/ml) and 100 mM NaCl. Substrate syringe contained 100 mM NaCl, 20 µM [γ-32P]ATP, and 1 mM KCl. In addition, all enzyme and substrate syringes in this and subsequent figures contained 60 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2.5 mM dithiothreitol and 3 mM MgCl. Curve drawn through P_i data is from a computer simulation using the values in Line 2, Table II. Curve through E-P data was drawn by eye.

<table>
<thead>
<tr>
<th>[K^+]</th>
<th>ΔP</th>
<th>P_i burst</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>nmol/mg</td>
<td>nmol/mg</td>
<td>nmol/mg/s</td>
</tr>
<tr>
<td>0</td>
<td>0.41</td>
<td>-0.04*</td>
<td>4.31</td>
</tr>
<tr>
<td>0.5</td>
<td>0.42</td>
<td>0.14</td>
<td>5.72</td>
</tr>
<tr>
<td>0.75</td>
<td>0.32</td>
<td>0.42</td>
<td>7.01</td>
</tr>
<tr>
<td>1.0</td>
<td>0.26</td>
<td>0.46</td>
<td>6.66</td>
</tr>
<tr>
<td>5</td>
<td>0.19</td>
<td>0.67</td>
<td>8.33</td>
</tr>
<tr>
<td>20</td>
<td>0.20</td>
<td>0.68</td>
<td>6.53</td>
</tr>
</tbody>
</table>

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*Extrapolated burst size has a negative value due to a short lag time prior to release of product.
phosphoenzyme was measured by mixing enzyme suspended in 100 mM Na⁺ with 20 mM K⁺ for various times prior to the addition of ATP. The decrease in the level of E-P measured 25 ms after the addition of ATP followed first order kinetics with a rate of 1.5 s⁻¹. This transformation appeared to be too slow to be accounted for by a normal enzymatic pathway, and suggested the possibility of a sequestered K⁺ site, perhaps caused by the presence of vesicles in the enzyme preparation. We tested this possibility with an experiment using valinomycin (18). When the enzyme was pretreated with Na⁺ and K⁺ added later, an increase in ATPase activity (from 1.17 to 1.7 pmol m⁻¹ mg⁻¹) occurred in the presence of valinomycin. In addition, the steady state E-P level was reduced from 0.09 to 0.04 μmol/mg. It appears from these results that the slow component of E-P seen in this enzyme is caused by the presence of “inside out” vesicles. When Na⁺ and ATP are added, the vesicular enzyme is rapidly phosphorylated on the outside, but cannot be dephosphorylated until sufficient K⁺ diffuses to the interior of the vesicles.

When Na⁺ and K⁺ were present before phosphorylation, the presence of valinomycin did not reduce the amount of stable E-P measured (about 50% of the total). Thus, there appear to be at least two types of stable E-P, one due to vesicles, sensitive to valinomycin, and the other which is insensitive.

**Analysis of Rate Constants**—Although Mähr and Lindahl (12) were able to simulate their results using a simple kinetic scheme, others (5, 11, 19, 20) have reported finding evidence for more than one pathway of ATP hydrolysis. In order to determine whether phosphoenzyme turnover is sufficient to account for P, release in the experiments shown in Fig. 2, the data were simulated using a reduced version of Mechanism II:

\[ E_1 + S \rightleftharpoons E_1S \rightarrow E_1 - P \rightarrow E_2 + P, \Rightarrow E_1 \]  

(1)

For the reasons given by Lowe and Smart (11), the ADP-sensitive form of the phosphoenzyme has been omitted and phosphorylation and dephosphorylation treated as irreversible reactions. The solid lines drawn through the P, data and dashed line, representing E-P formation for the case where Na⁺ and K⁺ are both present initially, were generated by the computer using the values for the rate constants given in Table II. As seen in Fig. 2, the computed E-P time course approximately satisfies the data for the case where the enzyme is first incubated with Na⁺ plus K⁺ but is significantly less than the observed E-P level when K⁺ is omitted from the enzyme medium. Comparison of the predicted E-P level in Fig. 2 with the measured amount of rapidly decaying species (Fig. 3) shows that they are similar. The difference between the predicted and observed E-P in the Na⁺-pretreated enzyme is mainly due to the presence of vesicles. The P, data for the Na⁺-preincubated enzyme can be simulated using the same rate constants that were used to simulate the result with the (Na⁺ plus K⁺)-preincubated enzyme, but with a smaller site concentration.

From the simulation in Fig. 2, at 20 mM K⁺, the dephosphorylation rate constant, \( k_5 \), is 350 s⁻¹, while the rate-limiting conformational transition between \( E_2 \) and \( E_1 \) is approximately 25 times slower (Table II). At 0.5 mM K⁺, the conformational transition rate increases and the dephosphorylation rate decreases relative to 20 mM K⁺. This assumes that all of the E-P is turning over at a reduced rate, which may not be the case if K⁺ is in rapid equilibrium with the high affinity sites (\( K_{6,6} \approx 0.6 \text{ mM} \)). In that event, only a small fraction of the sites are occupied and turning over rapidly, while the remainder turn over at the K⁺-independent rate.

It is consistent with the evidence showing that K⁺ has reciprocal effects on the dephosphorylation and subsequent conformational change (13), that \( k_5 \) had to be decreased and \( k_4 \) increased in order to fit the results of an experiment done in the absence of added K⁺. In addition, to prevent the predicted rate and extent of E-P formation from becoming too large at zero K⁺, further adjustments in the model parameters are necessary: either \( k_1 \) or \( k_2 \) must be decreased, while in addition the site concentration must be decreased, \( k_1 \); must be increased, or the phosphorylation reaction must be made irreversible. This suggests that the presence of K⁺ has an effect on either substrate binding or subsequent phosphorylation.

**Table II**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constants for E-P and P, formation of the (Na⁺ + K⁺)-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>( k_1 ), ( k_2 ), ( k_3 ), ( k_4 ), ( k_5 )</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.5, 0.9 x 10⁻⁴, 35, 150, 17, 18</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.7, 1.2 x 10⁻⁴, 35, 150, 28, 14</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>1.08, 1.2 x 10⁻⁴, 35, 150, 350, 12</td>
</tr>
</tbody>
</table>

In addition to the ligands indicated, all solutions contained 3 mM MgCl₂, 60 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, and 0.1 mM EDTA. In all simulations shown here \( k_5 \), \( k_6 \), and \( k_7 \) were set at 0.

**Experimental conditions**

<table>
<thead>
<tr>
<th>Syringe I</th>
<th>Syringe II</th>
<th>nmol/mg</th>
<th>k⁺</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>20 μM [γ-³²P]ATP</td>
<td>100 mM NaCl</td>
<td>0.5</td>
<td>0.9 x 10⁻⁴</td>
<td>35</td>
<td>150</td>
</tr>
<tr>
<td>Enzyme</td>
<td>20 μM [γ-³²P]ATP</td>
<td>100 mM NaCl</td>
<td>0.7</td>
<td>1.2 x 10⁻⁴</td>
<td>35</td>
<td>150</td>
</tr>
<tr>
<td>Enzyme</td>
<td>20 μM [γ-³²P]ATP</td>
<td>100 mM NaCl</td>
<td>1.08</td>
<td>1.2 x 10⁻⁴</td>
<td>35</td>
<td>150</td>
</tr>
</tbody>
</table>

In addition to the ligands indicated, all solutions contained 3 mM MgCl₂, 60 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, and 0.1 mM EDTA. In all simulations shown here \( k_5 \), \( k_6 \), and \( k_7 \) were set at 0.
In simulations of this type, rates assigned are highly dependent upon each other, and data may be fitted satisfactorily with more than one set of rate constants. Several of our assigned rate constants have been taken from a previous study on the Electrophorus (Na⁺ + K⁺)-ATPase (9), specifically, the substrate binding and subsequent phosphorylation rates. A minimum site concentration can be obtained from the amount of E-P which can be measured under conditions of maximal phosphorylation at 0°C. However, the site concentration (Eₚ) and the dephosphorylation rate (k₄) can be varied reciprocally to fit the same P, data, as can Eₚ and k₄, the rate of conversion of Eₚ to Eₛ. The former set has a large effect on the P, burst, whereas the latter changes mainly the steady state rate. Thus, values of k₄, k₅, and Eₚ are somewhat indeterminate, and within certain boundaries, k₄ and k₅, can be varied reciprocally with Eₚ to fit the experimental results about equally well.

To determine the amount of the K⁺-resistant stable phosphoenzyme (Eₛ-P) and the rate at which it was being dephosphorylated we fitted the data in Fig. 3 to the sum of two exponentials:

\[ Eₛ-P = [Eₛ-P] \exp(-k₁t) + [Eₛ-P] \exp(-k₂t) \]

where Eₛ-P was the total amount of E-P present 116 ms after the phosphorylation was started, Eₚ-P was the amount that dephosphorylated rapidly, and Eₛ-P the amount that dephosphorylated slowly upon addition of unlabeled ATP to prevent rephosphorylation. The results of this fitting procedure (Table III) are similar to those obtained by Mårdh in similar experiments (7), but are too slow to account for the rate of formation of the P, burst. This could mean either that there is a pathway not involving an E-P intermediate, as suggested by others (5, 11, 19, 20) or that the observed rate of dephosphorylation appears to be slower than the true rate because of continued formation of Eₛ-P from Eₛ-S after addition of excess unlabeled ATP.

We tested the feasibility of the latter hypothesis by computer simulations of the data in Fig. 3 using the rate constants in Table II. After subtracting the estimated contribution of Eₛ-P obtained from Table III, decay of Eₛ-P was simulated using the rate constants for conditions where Na⁺ and K⁺ are both present initially, but setting k₅ to zero because rephosphorylation of the enzyme was prevented in this experiment. Curves shown in Fig. 3 are the sums of Eₛ-P and Eₛ-P for each condition. Amounts of Eₛ-P were obtained from Table III, and the rate constants for decay obtained from Table II. Solid lines in the figure represent the best simulated fits to the data, while dashed lines indicate fits obtained when k₅ is varied. It can be seen from this figure that use of exponential decays to determine dephosphorylation rates can be deceptive, as in both cases dephosphorylation rates about 3 times higher than those determined in this way can be used to fit the data if the presence of Eₛ-S is taken into account. It is also apparent from this figure that the simulated time course is rather insensitive to changes in the dephosphorylation rate.

**Table III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Eₛ-P</th>
<th>k₁</th>
<th>Eₛ-P</th>
<th>k₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Na⁺, E) + (K⁺, [³²P]ATP) + (ATP)</td>
<td>0.0189</td>
<td>138</td>
<td>0.0878</td>
<td>3.3</td>
</tr>
<tr>
<td>(Na⁺, K⁺, E) + [³²P]ATP + (ATP)</td>
<td>0.0258</td>
<td>83</td>
<td>0.027</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Dephosphorylation in Presence and Absence of K⁺**—Fig. 4 shows two experiments in which phosphoenzyme was formed in the presence of 100 mM Na⁺ and 10 μM [³²P]ATP and dephosphorylated by the addition of 0.5 mM unlabeled ATP. Lower curve (○) shows enzyme phosphorylated for 116 ms in the presence of 100 mM NaCl and 10 μM [³²P]ATP and dephosphorylated by the addition of 5 mM KCl.

**Dephosphorylation in Presence and Absence of Na⁺**—Fig. 5 shows two experiments in which phosphoenzyme was formed in the presence of 100 mM Na⁺ and 10 μM [³²P]ATP and dephosphorylated by the addition of 5 mM KCl.

**Short Term Effects of K⁺ and Na⁺ Exposure**—Inhibitory effects of preincubation with potassium without sodium both on the rate of phosphorylation and the initial release of P, have been reported by several laboratories (8, 11, 12). When we attempted to examine this phenomenon in the eel enzyme in single mixing experiments by premixing the enzyme with K⁺ and Mg²⁺, then adding Na⁺ and ATP to start the reaction, we found that the results depended on the length of time which had elapsed between combining the enzyme with K⁺ and the addition of ATP. Longer times (minutes) produced a larger depression of E-P and an enhancement of P, measured at fixed times after addition of Na⁺ and ATP due, in part, to the presence of vesicles (but see below). To avoid this problem we resorted to the use of double mixing experiments so that the enzyme could be preincubated with K⁺ for a fixed short time prior to the addition of the other ligands. Fig. 5 shows the results of two double mixing experiments in which the effects of changing the order of addition of Na⁺ and K⁺ on phosphorylation and P, release were compared. Addition of K⁺ before Na⁺ and ATP reduced the rate of formation of E-P.

**Fig. 4.** Effect of potassium ions on dephosphorylation of E-P. Upper curve (□) shows enzyme phosphorylated for 116 ms in the presence of 100 mM NaCl and 10 μM [³²P]ATP and dephosphorylated by the addition of 0.5 mM unlabeled ATP. Lower curve (○) shows enzyme phosphorylated for 116 ms in the presence of 100 mM NaCl and 10 μM [³²P]ATP and dephosphorylated by the addition of 5 mM KCl.
and depressed the Pi burst. Comparatively high rates of phosphorylation and Pi release after Na+ preincubation are believed to reflect stabilization of the dephosphoenzyme in the E1 conformation, whereas slow rates of phosphorylation and initial Pi release are believed to arise from K+-induced stabilization of the dephosphoenzyme in the E2 conformation (11-13).

The interpretation of the phosphoenzyme data obtained in these experiments is complicated by the presence of vesicles, however. The simulation (dashed line) in Fig. 5 shows the amount of E-P required to explain the phosphate data obtained, using the rate constants in Table II, and initial conditions such that 90% of the enzyme was in E2 and 10% in E1. Of note is the fact that the initial rise in phosphoenzyme is much more rapid in the simulated than the observed data. This is because in the simulation, phosphoenzyme is being dephosphorylated rapidly as it is formed, and rapidly reaches the steady state. In the experimental data, much of the phosphoenzyme remains phosphorylated during the time of the experiment due to the lack of K+ on the inside of the vesicles.

An experiment in which enzyme was preincubated briefly with 30 mM K+ and phosphorylated in the presence of 100 mM Na+ and 10 μM ATP was performed. Two components were observed in the phosphoenzyme formation, a small fast component comprising about 15% of the total E-P, and a slower rise which had a rate (kapp = 8 s⁻¹) which agrees closely with the simulated rate of conversion of E2 to E1, using Mechanism II. Since the total E-P comprises less than 20% of the estimated site concentration, it appears that not more than 5% of the enzyme is in the E2 conformation under these conditions. A simulation of the Pi data gives results which are in agreement with this estimate. Comparison of these data to those obtained at 20 mM K+ shows that increasing K+ decreases the amount of E1 but does not change the rate of conversion of E2 to E1.

**Rates of Conversion between E1 and E2** - The time course of the transition between E1 and E2 upon exposure to K+ was examined by measuring the effects of varying short incubation times with 20 mM K+ (Fig. 6). Increased K+ incubation times resulted in decreased levels of both E-P and Pi. The reduction in the amount of Pi released after increased K+ preincubation time represents depression of the initial phosphate burst, presumably due to the conversion of E1 to E2. (For a more detailed treatment of this, see “Appendix.”) This conversion appears to follow first order kinetics with a pseudo-first order rate constant of 52 s⁻¹ at 20 mM K+ (a second experiment gave a rate of 55 s⁻¹). This is close to the maximum rate of conversion since a similar experiment at 30 mM K+ gave a rate of 60 s⁻¹. Experiments conducted at lower K+ concentrations took longer to reach equilibrium, however. For this reason we investigated the K+ dependency of the effect in another experiment, varying K+ and using a fixed preincubation time of 837 ms (Fig. 7). The K0.5 for this inhibitory effect is about 2.5 mM, much higher than that for the stimulatory effect seen when K+ is added with Na+. The effect also appears to be highly cooperative, with a Hill coefficient of about 3.

Reconversion of slowly reacting E2-K to a more rapidly reacting form of the enzyme occurred upon addition of Na+ to the enzyme mixture prior to the addition of ATP. Enzyme was mixed with KCl, and NaCl added within 1 min (to avoid complicating the results with effects of a long term preincubation). After preincubation with Na+ for various times, ATP was mixed with KCl, and NaCl added within 1 min (to avoid complicating the results with effects of a long term preincubation). After preincubation with Na+ for various times, ATP was added to the E-P + Pi mixture, and the enzyme mixture prior to the addition of ATP. Enzyme was mixed with KCl, and NaCl added within 1 min (to avoid complicating the results with effects of a long term preincubation). After preincubation with Na+ for various times, ATP was added and E-P and Pi release were measured after 50 ms. The time course of increase in the Pi burst followed first order kinetics, with an apparent rate constant of 6.3 s⁻¹. A rate constant of 5.5 s⁻¹ was obtained in a second experiment.
This rate is slightly lower than the rate (8 to 12 s⁻¹) used to fit single mixing experiments (e.g. Fig. 2). A possible explanation for this is that the presence of ATP stimulates the conversion of $E_2$ to $E_3$ (13). An experiment similar to the one described above was done, but with 100 µM ATP added with the NaCl, and MgCl₂ omitted until the final stage of the reaction. EDTA (1 mM) was added in the early stages to prevent hydrolysis. Accumulation of P₃ 25 ms after addition of MgCl₂ was maximal at the shortest time of incubation with NaCl and ATP, indicating a conversion of $E_1$ to $E_2$ which was much more rapid than that in the absence of ATP.

**Long Term K⁺ Exposure**—Prolonged exposure to 20 mM KCl before addition of NaCl and ATP restores an initial rapid rate of phosphorylation and P₃ liberation, but reduces the steady state level of phosphoenzyme (to 20% of original) and magnitude of the burst (to 50% of original) (Fig. 8). The steady state rate of ATP hydrolysis, however, remains the same.

To examine the time dependence of this phenomenon, enzyme was exposed to 20 mM K⁺ for various time periods, then incubated with Na⁺ and ATP for 50 ms. This K⁺-induced transformation involved an initial decrease in the transient hydrolytic activity followed by an increase which was complete within 1 h. Phosphoenzyme levels were significantly reduced and remained low. Part of this reduction in phosphoenzyme is due to the vesicles which were described earlier. However, the phosphoenzyme in this experiment is reduced about 25% compared to that in a similar experiment where Na⁺ and K⁺ are present initially. Dephosphorylation of the E-P present after long term K⁺ preincubation revealed that the rapidly and slowly dephosphorylating components were present in about the same proportion (50%) as the enzyme which had been pre-exposed to Na⁺ and K⁺ (Fig. 3). The time course of formation of this E-P is rapid, similar to the rate expected if vesicles were not present (inset, Fig. 5).

If this effect were only due to the presence of vesicles, however, partial restoration of the P₃ burst would not be expected to occur. Dilution from 20 mM to 1 mM K⁺ several minutes before starting the experiment, or the addition of 100 mM Na⁺ prior to ATP restored the P₃ burst to its original magnitude, but failed to elevate the E-P level. Valinomycin also failed to alter this long term depression of the initial reactivity. This is consistent with the hypothesis that these two effects are independent of one another. The fall in E-P level can be most easily explained by diffusion of potassium into vesicles, so that $E_3$ can be dephosphorylated. The decrease in P₃ liberated during the early burst suggests that after an initial relatively rapid conversion of $E_3$ to $E_2$ by addition of K⁺, there is a much slower effect of K⁺ that converts about half the enzyme back to a form that rapidly hydrolyzes ATP.

**Discussion**

Under conditions where Na⁺ or Na⁺ plus K⁺ are present initially or following brief exposure to K⁺ in the absence of Na⁺, phosphorylation of the (Na⁺ + K⁺)-ATPase is necessary for ATP hydrolysis. This conclusion is supported by the following observations: (a) The results of the single (Figs. 1 and 2) and double (Fig. 3) mixing experiments fit a simple model for ATP hydrolysis which includes enzyme phosphorylation as an intermediate step, and the results of both types of experiments are satisfied by a single choice of rate constants. (b) As K⁺ is increased, the initial P₃ burst and steady state P₃ production increase and phosphoenzyme levels decrease in a reciprocal fashion (Table I). This suggests that E-P₃ is breaking down more rapidly to produce the increased phosphate observed. (c) Following brief exposure to K⁺ without Na⁺, both E-P and P₃ are transiently reduced when Na⁺ and ATP are added (Fig. 6). Also, under conditions where E-P₃ formation is slowed, transient P₃ production is greatly diminished or absent (Fig. 5).

Our dephosphorylation data can be fitted with a model in which the conformational transition $E_3$-P → $E_2$-P is very fast, and dephosphorylation of the latter occurs at a rate of about 300 s⁻¹. Direct measurement of the rate constant for dephosphorylation of $E_2$-P is difficult because the velocity of the reaction is near the limit of resolution in these experiments. Initial decay of $E_3$-P will fit a rate of about 300 s⁻¹, but the rate may be faster, in which case the rate that we measure may be that of the conformational transition between $E_3$-P and $E_2$-P, with $E_3$-P breaking down too rapidly to be measured. This would mean that under steady state conditions, the species which accumulates is $E_1$-P. However, the observations that there is very little ATP-ADP exchange under these conditions (21) and that formycin diphosphate is rapidly dissociated from the enzyme (22) argue against this possibility.

The effects observed when electroplax (Na⁺ + K⁺)-ATPase is preincubated with Na⁺ or K⁺ closely parallel the behavior found in mammalian enzymes in similar experiments. When the enzyme is first treated with Na⁺, and K⁺ is added simultaneously with ATP, there is a rapid rate of phosphorylation and a transient burst of product formation. Because pretreatment with Mg⁺ alone, or with Na⁺ and K⁺ together, produces similar behavior, it appears that the enzyme is primarily in the $E_3$ form under these three conditions. When K⁺ is added briefly to the enzyme without Na⁺, however, a slow rate of phosphorylation is observed and the burst is greatly reduced or absent. These results can be explained by assuming that the unphosphorylated enzyme ordinarily exists in the $E_3$-state, and only when K⁺ is present without Na⁺ is $E_2$ favored.

In addition to the rapidly decaying phosphoenzyme, the time course of dephosphorylation also contained a slowly decaying component ($k_{\text{app}} = 2$ to 4 s⁻¹) which accounted for less than 2% of the overall enzyme activity. By treatment with valinomycin or early exposure to K⁺, the slowly decaying phosphoenzyme could be further resolved into K⁺-sensitive and K⁺-insensitive components, the former presumably reflecting the presence of sealed vesicles with dephosphorylation sites sequestered. The presence of vesicles in the preparation has allowed us to measure phosphorylation rates starting from either the $E_3$ or $E_2$ conformations which are close to the true values for these rates. Had vesicles not been present, the observed phosphorylation rate starting from $E_2$ would have been much higher due to subsequent rapid dephosphorylation.

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**Fig. 8. Effect of long term pre-exposure to KCl in the absence of NaCl on phosphate release and E-P formation.** Enzyme was premixed with 20 mM KCl 30 min before starting the reaction by the addition of 10 µM [γ-³²P]ATP and 100 mM NaCl. Inset shows phosphoenzyme formation on expanded scale.
The change in magnitude of the initial phosphate burst can be used to indirectly monitor the conformational transitions between $E_1$ and $E_2$ (see "Appendix"). The time-dependent changes in the $P_i$ burst (Fig. 6) can be described in terms of a minimum mechanism:

$$E_1 + K \rightarrow E_2 \rightarrow E_1 + \text{Na}$$

(V)

which assumes that Na$^+$ and K$^+$ bind preferentially to $E_1$ and $E_2$, respectively. Upon addition of K$^+$ to microsomes suspended in a medium containing either Mg$^{2+}$ or no cations other than the buffer, $E_1$ was converted to $E_2$ with a maximum rate of 55 to 60 s$^{-1}$. Reconversion to $E_1$ occurred at a rate of 6 s$^{-1}$, a value which compares favorably to the phosphorylation rate of 8 s$^{-1}$ measured after brief K$^+$ preincubation. ATP appears to stimulate this transition, shown in an experiment in which ATP was added with the Na$^+$.

Taken together, these facts indicate some modification of the scheme shown above. If the relaxation rate of the system to $E_2 + K$ is 55 s$^{-1}$ when K$^+$ is added, but the rate of recovery to $E_1$ is only 6 s$^{-1}$, the system must be more complex, or the equilibrium would lie far to the left, even in the absence of K$^+$. Assuming a mass action effect of Na$^+$ to prevent reformation of $E_2$, the forward rate of the reaction is 6 s$^{-1}$. The reverse rate must be 49 s$^{-1}$ to give the observed rate of transition from $E_1$ to $E_2$. If ion binding and dissociation rates are rapid, the following scheme could account for the results:

$$E_1 \rightarrow E_1 + \text{Na}$$

(VI)

$$E_1 + K \rightarrow E_1 + \text{Na}$$

(VII)

$$E_1 + K \rightarrow E_2 + K$$

(VIII)

$$E_2 + K \rightarrow E_1 + \text{Na}$$

(VIII)

$$E_2 + K \rightarrow E_1 + \text{Na}$$

(VIII)

In this model, the $E_2 = E_1$ equilibrium is poised to the right, while the $E_2 + K = E_1 + K$ equilibrium lies far to the left, with forward and reverse rates of 6 and 49 s$^{-1}$, respectively. This model implies that K$^+$ can bind to the $E_1$ conformation. The cooperativity shown by the data (Fig. 7) indicates that more than one K$^+$ site is involved, and the apparent dissociation constant (2.5 mM) indicates that these sites are relatively weak.

Other laboratories have measured the conformational changes between these species, but under slightly different conditions. Karlish and Yates (23) measured the forward rate directly using tryptophan fluorescence changes in mammalian kidney enzyme, and found that in the absence of ATP the rate, 0.26 s$^{-1}$, was much slower than that which we measured in the Electrophorus enzyme. The Electrophorus enzyme also requires a much higher K$^+$ concentration, 2.5 mM as opposed to 50 mM for the kidney enzyme, to obtain a half-maximal transition to $E_2 + K$ in Na$^+$-free medium. However, their measurements were carried out in the absence of Mg$^{2+}$. Using formycin nucleotides, Karlish et al. (24) measured the reverse rate, and found it to be about 100 s$^{-1}$ at 15 mM K$^+$, higher than the eel enzyme exhibits at 30 mM. In addition, the rate in their experiments did not appear to be approaching saturation. This difference, as well as the higher K$^+$ concentration required for half-maximal conversion to $E_2 + K$, is probably explained by their finding that Mg$^{2+}$ decreases the transition rate from $E_1$ to $E_2 + K$ (24). The difference in forward rates, however, may represent an intrinsic difference in the enzyme preparations, as the absence of Mg$^{2+}$ in the experiments of Karlish et al. did not appear to affect the forward rate.

Accumulation of the enzyme in the $E_2 + K$ form should not result in an initial phosphate burst; however, such a burst was observed following prolonged exposure to K$^+$ in the absence of Na$^+$. The relationship between P production and E-P formation under these conditions is unclear. If E-P turnover is responsible for the burst, an E-P overshoot would be expected to appear during the initial stages of the reaction. That one was not detected, and that too little phosphoenzyme is observed in these experiments to account for the P$_i$ release in the burst with a reasonable rate of dephosphorylation, suggests that phosphoenzyme formation and the appearance of the P$_i$ burst are manifestations of different catalytic pathways of the enzyme.

Thus, following short term exposure to K$^+$, all sites behave as if they were initially stabilized in the $E_2 + K$ conformation and react slowly to form $E_0$, which is rapidly phosphorylated by ATP. In the presence of Na$^+$, Na$^+$ plus K$^+$, or Mg$^{2+}$, all sites appear to be in $E_1$. After prolonged exposure to K$^+$, some sites appear to be present as $E_2$, while the remainder are catalytically active, but apparently hydrolyze ATP without formation of phosphoenzyme, at least during the initial cycle of activity. If this behavior involves an alternate pathway of the ATPase, it appears to be a unique situation, since results obtained from other experiments can be adequately explained by a single pathway. Moreover, the mechanism is probably of limited physiological significance since Na$^+$, which is present in living systems, would prevent such effects.

**APPENDIX**

It may not be immediately apparent how measuring the rate of change in magnitude of the initial phosphate burst upon preincubation with various ligands can accurately reflect a change in the distribution between $E_1$ and $E_2$ prior to ATP addition, such as the experiment shown in Fig. 6. The fact that ATP stimulates the forward reaction, at least at high ATP concentrations (13, 24, and this paper) might seem to invalidate this approach. Computer simulations using the simple four-state model which we have used throughout much of this paper

$$E_1 \leftrightarrow E_2 \leftrightarrow E_1 \cdot K \leftrightarrow E_2 \cdot K$$

(with plausible rate constants) demonstrate that this method does indeed measure the rate of change of $E_2$ to $E_1$ even when addition of ATP has an additional effect. Although a more formal proof is possible, we will demonstrate here that for several sets of rate constants the change in the initial phosphate burst directly reflects the shift in equilibrium between $E_1$ and $E_2$ prior to the addition of ATP.

Computer simulations were done using MLAB (17) and integrating the following model:

$$dA = -k_1 [A] + k_2 [B] + k_3 [D]$$

$$dB = -k_1 [B] + k_2 [C] + k_1 [A] - k_3 [B]$$

$$dC = -k_3 [C] + k_4 [B] - k_2 [C]$$

$$dD = -k_3 [D] + k_4 [C]$$

$$dP = k_1 [D]$$

where $A = E_1$, $B = E_2$, $C = E_1 \cdot ATP$, $D = E_2 \cdot P$, and $P = P_i$. Prior to the addition of ATP, $E_1$ and $E_2$ will exist in a distribution determined by the ligands present, their time of interaction with the enzyme, and the forward and reverse rate constants, $k_1$ and $k_2$. Because step 1 is the rate-limiting step in the overall reaction, the amount of P$_i$ released rapidly in the initial burst will depend upon the amount of $E_2$ present at the time of ATP addition. Enzyme which is in the $E_0$ form at this time will not contribute to the burst.

As shown in Table IV, the amount of P$_i$ measured in
experiments of this type will vary, but the rate of transformation from one $E_i \rightarrow E_f$ equilibration to another will remain constant. In Case 1, the enzyme is presumed to exist entirely as $E_f$ in the absence of $Na^+$ and $K^+$. However, this assumption is not essential for the results of these simulations to be valid. When $20 \text{ mM } K^+$ is added without $Na^+$ (simulating the experiment shown in Fig. 6), the equilibrium becomes $E_f \rightarrow E_1$, and the enzyme is converted to the $E_2$ form, with a relaxation rate of $55 \text{ s}^{-1}$. After various short times, $Na^+$ and ATP are added simultaneously and allowed to interact with the enzyme for a fixed time (e.g. $50 \text{ ms}$) using a set of rate constants presumed to exist after ATP and $Na^+$ addition. It can be seen that even if $k_1$ is then increased from 6 to $50 \text{ s}^{-1}$ (compare Cases 1a and 1b), the apparent relaxation rate reflects conditions before the addition of ATP. $k_2$ has been reduced to zero upon the addition of ATP, assuming a mass action effect on $Na^+$, or ATP, or both, to prevent reversal of the reaction. This assumption seems reasonable, since the forward rate of conversion ($E_2 \rightarrow E_1$) is only $6 \text{ s}^{-1}$. The relaxation rate reflects the sum of the forward and reverse rates and the reverse rate must clearly be very low, or the transformation to $E_2$ to $E_1$ would not occur. Although the magnitude of the change is less in case 1b, the rate is similar, and the time course of the change remains the same. Of course, if $k_2$, becomes too large no burst will be observed.

A similar argument can be made for the reverse case ($E_2 \rightarrow E_1$ upon addition of $Na^+$), as shown in Cases 2a and 2b. In this way we can place constraints upon the sum of the forward and reverse constants between the two intermediates which we are observing in Fig. 6. Values of $P_i$ obtained at longer times will reflect true differences in the burst size, while those obtained at shorter times will be proportionally similar, but not reflect the true burst size.

REFERENCES


