Low Affinity Acceleration of the Phosphorylation Reaction of the Na,K-ATPase by ATP*

(Received for publication, June 7, 1993, and in revised form, September 8, 1993)

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The maximum rate of phosphorylation ($r_m$) of a highly purified Na,K-ATPase from red outer medulla of pig kidney was measured at 25 °C as a function of ATP concentration in media with Mg$^{2+}$, Na$^+$, and no K$^+$. When $r_m$ was plotted as a function of the concentration of ATP a biphasic response was observed with a hyperbolic component of high affinity ($K_m = 15.7 \pm 2.6 \mu M$) and low velocity ($r_m_{\text{max}} = 460 \pm 40 \text{ nmol of P}/(\text{mg of protein-s})$) plus a parabolic component which showed no saturation up to 1000 μM ATP, concentration at which $r_m$ was 1768.1 ± 429.6 nmol P/(mg protein-s) (mean ± S.E.; n = 3). This low affinity effect of ATP on the rate of phosphorylation disappeared when the Na,K-ATPase underwent turnover in medium without K$^+$ suggesting that, like superphosphorylation (Peluffo, R. D., Garrahan, P. J., and Rega, A. F. (1992) J. Biol. Chem. 267, 6596–6601), it required the enzyme to be at rest.

This property of the Na,K-ATPase was not predicted by the Albers-Post reaction scheme. The observed behavior of the enzyme could be simulated by a scheme that involves a resting enzyme ($E_r$) functionally different from $E_i$ or $E_s$, which is able to bind three molecules of ATP, one with high and two with low affinity, and that after phosphorylation is converted into the phosphointermediates that are generally considered to participate in the reaction cycle described by Albers and Post.

It is generally accepted that phosphorylation of the Na,K-ATPase by ATP requires the combination of one molecule of the nucleotide with the $E_i$ conformer of the enzyme followed by the transfer of the γ-phosphate of ATP to the β-carboxylate group of an aspartyl residue of the α-subunit of the enzyme. As binding of ATP precedes phosphorylation, the apparent rate constant of phosphorylation has to be extrapolated to infinite ATP concentration to estimate the true rate constant of this reaction. Values from the literature, vary because of the Albers-Post reaction scheme. The observed behavior of the enzyme could be simulated by a scheme that involves a resting enzyme ($E_r$) functionally different from $E_i$ or $E_s$, which is able to bind three molecules of ATP, one with high and two with low affinity, and that after phosphorylation is converted into the phosphointermediates that are generally considered to participate in the reaction cycle described by Albers and Post.

at 100 μM ATP instantaneous with respect to the rate of phosphorylation.

In a previous paper (Peluffo et al., 1992) we showed that as the concentration of ATP tends to infinity, the maximum level of phosphorylation tends to a value that is at least twice the maximum binding of ouabain, a phenomenon that we called superphosphorylation. Superphosphorylation was transient and so fast that it made it difficult to measure its initial reaction. The phenomenon was observed only in the enzyme at rest and disappeared when the enzyme underwent turnover.

The present work was aimed to study the effect of ATP on the rate of phosphorylation under conditions that make the Na,K-ATPase able to undergo superphosphorylation. Experiments were performed with a highly purified Na,K-ATPase prepared by zonal centrifugation to take advantage of the fact that this preparation showed a relatively low phosphorylation rate thus making possible a more accurate measurement of this parameter. It was found that when the concentration of ATP increased above 300 µM the rate of formation of the phosphoenzyme increased several times along a parabolic curve.

MATERIALS AND METHODS

Enzyme Source—Na,K-ATPase (specific activity, 25.7 µmol of P/ (mg protein-min) measured according to Esmann (1988)) from pig kidney red outer medulla purified by zonal centrifugation (Jørgensen, 1974) and kindly provided by Dr. J. Jensen from the Institute of Physiology, Århus University, Denmark, was used.

Phosphorylation of the Na,K-ATPase—Phosphorylation was carried out at 25 °C in a rapid mixing apparatus adapted for chemical quenching (Intermekron, Uppsala) (Mårth and Zetterqvist, 1974). The incubation buffer contained 150 mM NaCl, 0.2 mM EDTA, 40 mM imidazole-HCl (pH 7.5), and enough MgCl$_2$ to give a final concentration of 0.5 mM free Mg$^{2+}$ in all experiments (see below). The reaction was started by mixing the enzyme (10–15 µg of protein/ml) in 2 ml of incubation buffer with 2 ml of the same incubation buffer containing 50 µM ATP (to give the desired final concentration of the nucleotide. Phosphorylation was quenched by collecting the reaction mixture in 7 ml of an ice-cold solution of 11% (w/v) trichloroacetic acid, 50 mM H$_2$PO$_4$, and 10 mM ATP. The mixture was filtered through a 0.45-µm Millipore filter, and the insoluble material was washed three times with 10 ml of an ice-cold solution of 7% (w/v) trichloroacetic acid and 32 mM H$_2$PO$_4$. The filters with the denatured enzyme were dried and placed in scintillation vials containing 5 ml of 0.4% (w/v) 2,5-diphenyloxazole, 0.02% (w/v) 1,3-bis-2-(5-phenyloxazole) in toluene for liquid scintillation counting. The amount of phosphoenzyme was estimated from the difference of the radioactivity incorporated into protein in the incubation buffer mentioned above minus the radioactivity incorporated under identical conditions in the same incubation buffer without MgCl$_2$.

Protein concentration was determined by the method of Lowry et al. (1951), following precipitation with trichloroacetic acid and using bovine serum albumin as the standard as described by Jørgensen (1974).
The concentration of free Mg\(^{2+}\) was estimated from the total concentrations of ATP, EDTA, and magnesium using the equilibrium constants for the dissociation of Mg\(^{2+}\) from MgATP\(^{2-}\) and MgEDTA\(^{2-}\) and the set of microscopic dissociation constants of H\(^+\) from EDTA\(_{4-}\) and ATPH\(_{4-}\) given by Dawson et al. (1969).

Reagents—\([\gamma\text{-}^{32}\text{P}]\)ATP (specific activity \(\approx 1 \text{mCi/\mu mol}\)) was prepared according to Glynn and Chappell (1964) except that no unlabeled orthophosphate was added. \([\text{32P}]\)Orthophosphate was provided by Comisión Nacional de Energía Atómica (Argentina). Enzymes and cofactors for the synthesis of \([\gamma\text{-}^{32}\text{P}]\)ATP were from Sigma. Salts and reagents were of analytical reagent grade.

Model Simulations—Numerical simulations of the kinetic schemes were performed by using a finite differences-based method, one of the tools included in a computer program ("Plot") developed by Dr. Bliss Forbush III. Reactions involving the release of ADP and inorganic phosphate were considered irreversible.

Calculation of the Maximum Rate of Phosphorylation—The curves representing the time courses of phosphorylation in this study showed large variation in shape as the concentration of ATP changed. Nevertheless, the following empirical equation was found to fit the time courses at all the concentrations of ATP

\[
[EP] = \frac{a \cdot t + b \cdot t^2}{c + d \cdot t + e^2}
\]  
(Eq. 1)

where \([EP]\) is the concentration of phosphoenzyme and the coefficients \(a, b, c, d,\) and \(e\) are functions of the ATP concentration and of the rate constants of the reactions involved. Equation 1 is similar to that used by Ferdinand (1966) to describe non-hyperbolic kinetics in regulatory enzymes. Inspection of Equation 1 shows that, in going from \(t = 0\) to \(t = \infty\), \([EP]\) goes from 0 to \(b\). The use of Equation 1 allows a single function to be fitted to all the time courses thus allowing to standardize the calculation of the maximum rate of phosphorylation.

The maximum rate of phosphorylation \((r_m)\) at each ATP concentration was estimated from the maximum value of the first derivative of \([EP]\) with respect to time.

\[
r_m = \frac{d[EP]/dt}_{\text{max}}
\]  
(Eq. 2)

\[\begin{align*}
&\text{FIG. 1.} \quad \text{a, time courses of phosphorylation of Na,K-ATPase with} \\
&\quad 1 (\bullet), 5 (\odot), 15 (\times), 200 (\square), 500 (A), \text{or 1000} \, \text{mM} \, [\gamma\text{-}^{32}\text{P}]\text{ATP.} \\
&\quad \text{The symbols represent the mean of three measurements \pm S.E. When not} \\
&\quad \text{shown, error bars fall within the symbols. The curves represent} \\
&\quad \text{the best fitting of Equation 1 to the experimental data at each} \\
&\quad \text{concentration of ATP. The maximum rate of phosphorylation is} \\
&\quad \text{shown at each concentration.} \\
&\quad \text{The small filled circles indicate the position of the inflection} \\
&\quad \text{points of the curves at each concentration of ATP. Note that, for} \\
&\quad 500 \text{ and} \\
&\quad 1000 \, \text{mM ATP, the curves are concave downward from the origin. The} \\
&\quad \text{dotted straight lines are the tangents to the curves either at the} \\
&\quad \text{inflection points or at the origin. From their slopes,} \, r_m \, \text{was} \\
&\quad \text{estimated.} \\
&\quad \text{b, an enlargement of the initial part of the curves.} \\
&\quad \text{The small filled circles indicate the position of the inflection} \\
&\quad \text{points of the curves at each concentration of ATP. Note that, for} \\
&\quad 500 \text{ and} \\
&\quad 1000 \, \text{mM ATP, the curves are concave downward from the origin. The} \\
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&\quad \text{estimated.} \\
&\text{\footnotesize{1 B. Forbush III, unpublished data.}}
\end{align*}\]
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Effects of the Concentration of ATP on Time Course of Phosphorylation—The time course of phosphorylation was measured during a 2.5-58 ms time interval in media containing 1, 5, 10, 15, 20, 30, 50, 100, 200, 300, 400, 500, 600, 750, or 1000 μM ATP. For the sake of clarity only 6 out of the 15 measured time courses are shown in Fig. 1a together with the solutions of Equation 1 that give best fit to each set of data. At low ATP concentrations a lag was apparent that tended to disappear at higher concentrations of the nucleotide. As it was expected from previous results from this laboratory (Peluffo et al., 1992) at 500 μM ATP or more superphosphorylation became apparent. As a consequence of this the curves passed through a maximum before falling to the same steady-state level as the curves at lower ATP concentrations.

Results also show that the rate of phosphorylation increased with the concentration of ATP. This effect is more easily seen on the initial part of the curves as shown in Fig. 1b, in which only the continuous lines of Fig. 1a are plotted. The circles on the curves indicate the inflection points at which the curves changed from curved upward to concave downward. The straight lines are tangent to each curve at either these points or at the origin for those curves that did not display a lag. As described under “Materials and Methods” the slopes of these lines represent the maximum rate of phosphorylation (r_m) at each ATP concentration. In going

![Diagram](image-url)

**Scheme 1.** Reaction scheme of the Na,K-ATPase that takes into account the low affinity effect of ATP. The scheme is based on that proposed by us to simulate superphosphorylation (Peluffo et al., 1992). Addition and release of ligands are omitted. EP is for (E,P ↔ E,P). E is the enzyme at rest. Binding of ATP to E takes place at high and at low affinity sites. ATP as superscript of E is for ATP bound to high affinity sites and ATP as subscript is for ATP bound at low affinity sites. In this scheme two molecules of ATP bind to E, with low affinity and at random, and without interactions in affinity. For low affinity phosphorylation, a third molecule of ATP must be bound with high affinity to form the complex E_ATP. The form E_ATP that can lead to phosphorylation directly, is also able of binding two molecules of ATP. Partial dephosphorylation of E_P3 drives the ATPase into a cycle that follows the Albers-Post model. To perform the simulations, the rate parameters for the addition and release of ATP from E, at the high affinity sites were assumed to be 10 s⁻¹ m⁻¹ and 2 s⁻¹, respectively. The rate constant for the addition of ATP to E, at the low affinity sites was 1.6 s⁻¹ m⁻¹. These values are those in the literature for the binding and release of the nucleotide at the high and the low affinity site during steady-state Na,K-ATPase activity (for references see Rossi and Garrahan (1989)). However, the best value found for the rate constant for the release of ATP from the low affinity sites of E_ATP, E_ATPP, E_ATP, and E_ATPP was 3200 s⁻¹, giving a K_a value of 2 mM. The rate constants were set at 180 s⁻¹ for phosphorylation of E_ATPP, at 5000 s⁻¹ for phosphorylation of E_ATPP, and at 100 s⁻¹ for the dephosphorylation of E_P3. The values of the rate parameters for the steady-state reaction cycle were taken from the literature (see Rossi and Garrahan (1989)).

**Results**

**Effects of the Concentration of ATP on Time Course of Phosphorylation**—The time course of phosphorylation was measured during a 2.5-58 ms time interval in media containing 1, 5, 10, 15, 20, 30, 50, 100, 200, 300, 400, 500, 600, 750, or 1000 μM ATP. For the sake of clarity only 6 out of the 15 measured time courses are shown in Fig. 1a together with the solutions of Equation 1 that give best fit to each set of data. At low ATP concentrations a lag was apparent that tended to disappear at higher concentrations of the nucleotide. As it was expected from previous results from this laboratory (Peluffo et al., 1992) at 500 μM ATP or more superphosphorylation became apparent. As a consequence of this the curves passed through a maximum before falling to the same steady-state level as the curves at lower ATP concentrations.

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**FIG. 4.** Time courses of phosphorylation simulated according to Scheme 1 at the micromolar concentrations of ATP indicated on the curves. It was assumed that at the start of the reaction the enzyme was a mixture of equal amounts of E, and E_2.

Calculations were performed using Equation 1 with the best fitting values of the coefficients. When the curves showed a lag, r_m was calculated for the value of t at which (d²[EP]/dt²) = 0 and (d²[EP]/ dt²) < 0. When no lag was apparent, r_m had to be taken as the value of (d[EP]/dt) at t = 0, because the analytical maximum required t <
from 1 to 1000 μM ATP, $r_m$ (± standard error) increased from 32.2 ± 3.8 to 1768.1 ± 428.6 nmol P/(mg protein s).

**Maximum Rate of Phosphorylation as a function of the Concentration of ATP**—The values of $r_m$ for all the 15 concentrations of ATP mentioned above were plotted against the concentration of ATP with the results shown in Fig. 2. At low ATP concentrations, $r_m$ increased along a curve that tended to saturation (inset in Fig. 2) so that from 100 μM to 300 μM ATP, $r_m$ remained almost constant. At about 300 μM ATP, $r_m$ started to increase again, now along a parabolic curve that showed no signs of saturation up to the highest ATP concentration tested.

Best fit to the data (continuous line in Fig. 2) was obtained with the sum of a Michaelis-Menten-like term plus a term in which the concentration of ATP is raised to a power $n$, i.e.

$$r_m = \frac{r_{m,\text{max}} [\text{ATP}]}{K_m + [\text{ATP}]} + A \cdot [\text{ATP}]^n$$

where ($r_{m,\text{max}}$ is the maximum value of the hyperbolic component, $K_m$ is the concentration of ATP needed for half-maximal effect on $r_m$ at this component, and A is an arbitrary constant. Notice that the value of $r_m$ measured at 1000 μM ATP was about 4 times higher than the best fitting value for ($r_{m,\text{max}}$ given in the legend to Fig. 2. As it is shown in the inset, at concentrations of ATP up to 100 μM the data could be fitted solely by the Michaelis-Menten-like term of Equation 3.

**Effect of ATP on the Maximum Rate of Phosphorylation of the Enzyme Undergoing Turnover**—In the experiments described up to this point, phosphorylation was started by the addition of [γ-32P]ATP to Na,K-ATPase suspended in media without ATP so that the enzyme was initially at rest (not cycling). To verify whether this was needed for the expression of the low affinity effect of ATP on $r_m$, an experiment was performed in which the enzyme was preincubated with 10 μM unlabeled ATP in the incubation buffer at 25 °C during 2 min and then phosphorylated in media with either 15 or 505 μM [γ-32P]ATP. Results in Fig. 3 show that in the preincubated enzyme the effect of high ATP on the time course of phosphorylation disappeared since the curves at 15 and 505 μM ATP became almost identical in shape and their $r_m$ values (74.5 ± 4.9 and 93.5 ± 31.0 nmol of P/(mg of protein·s), respectively), were not significantly different from each other.

To discriminate whether the effect of preincubation was caused by the binding of ATP or if it also required the functioning of the Na,K-ATPase, a control experiment (not shown) was run in which the enzyme was preincubated with 5 mM ATP; 0.2 mM EDTA; 150 mM Na+ at 25 °C during 25 min before phosphorylation. Under these conditions, lack of Mg2+ prevented the functioning of the enzyme. Results indicated that, in contrast with what was observed during the experiment of Fig. 3, acceleration of $r_m$ by high ATP persisted. Therefore, the disappearance of the effect of high ATP on $r_m$ requires the Na,K-ATPase to undergo turnover.

Comparison of the results in Figs. 3 and 1a also show that at 15 μM ATP the time courses of the cycling and resting enzymes displayed a lag and that their $r_m$ values were 74.5 ± 4.9 and 206.1 ± 8.7 nmol of P/(mg of protein·s), respectively. Therefore, at low ATP, turnover did not alter the shape of the curve describing the time course of phosphorylation but reduced the value of $r_m$ by a factor of 2.8. In the experiment in Fig. 1a the enzyme was suspended in a Na+-rich medium so that most of it should be as $E_r$ at the start of the reaction whereas in the experiment in Fig. 3, according to the Albers-Post model, the enzyme must undergo dephosphorylation and the $E_r \rightarrow E_1$ transition before binding ATP. Under these conditions the steady-state levels of $E_1$ and therefore the rate of phosphorylation should be lower than in the resting enzyme. This is the most likely explanation for the decrease of $r_m$ in the enzyme undergoing turnover.

**Reaction Scheme to Simulate the Low Affinity Acceleration of Phosphorylation by ATP**—If this effect were caused by ATP accelerating any of the transitions among the conformers that, according to the Albers-Post model, participate in the reaction cycle of the Na-ATPase, then steady-state hydrolysis should exhibit a low affinity activation by ATP. Since this is not the case, a possible explanation of the phenomenon is that the resting enzyme could be functionally different from that undergoing steady-state turnover as we already proposed to account for superphosphorylation (Peluffo et al., 1992). To explain the shape of the curve relating $r_m$ to the ATP concentration at high concentrations of the nucleotide, the reaction scheme we had used to explain superphosphorylation was modified as shown in Scheme 1. The basic assumptions for Scheme 1 are: (a) in the Na,K-ATPase at rest ($E_r$), both high and low affinity sites for ATP coexist and become phosphorylated, and (b) the transition of the enzyme from the resting to cycling state requires phosphorylation, which is faster if $E_r$ simultaneously binds one molecule of ATP with high affinity and two with low affinity. The only difference between Scheme 1 and that for superphosphorylation is the inclusion of the low affinity binding of two, instead of one, molecules of ATP. This was necessary to account for the parabolic response of $r_m$ at high concentrations of ATP. The analytical solution of Scheme 1 showed that the parabolic component is the initial part of a sigmoidal curve which saturates at concentrations of ATP much higher than those used in the experiment in Fig. 2. Scheme 1 makes no assumptions on the number of sites for ATP in either α-subunit of the ATPase, since from the kinetic point of view it is irrelevant whether the sites for ATP coexist in the same α-subunit or in different α-subunits associated in an oligomer.

Fig. 4 gives the results of the simulations based on Scheme 1 of the time courses of [EP] at the concentrations of ATP used in Fig. 1, a and b. Comparison of the curves in Fig. 4 with those in Fig. 1b shows that this scheme correctly reproduced the experimental time courses of phosphorylation. The values of $r_m$ were estimated from the maximum value of the slope of the simulated data (see "Materials and Methods"). The simulated curve of $r_m$ as a function of the concentration of ATP up to 1000 μM (dashed line) was plotted in Fig. 5 together with the experimental curve in Fig. 2 (continuous line). It can be seen that the curves are very similar in shape. The two additional curves in Fig. 5 are those generated by either the Albers-Post model (dotted line) or the reaction scheme proposed by us (Peluffo et al., 1992) for superphosphorylation (dashed-dotted line). The Albers-Post model predicted a Michaelis-Menten-like dependence with $K_m$ near 20 μM. The scheme used for superphosphorylation showed biphatic behavior (Fig. 5) with a low affinity effect of ATP which does not fit with the experimental results since it corresponds to the initial part of a Michaelis-Menten curve with $K_m$ much higher than the highest ATP concentration tested. The inset in Fig. 5 shows that up to 30 μM ATP the curves for all the models tested were not distinguishable from each other. This was so because at low ATP concentrations only the high affinity sites for the nucleotide should be significantly occupied, making $E_r$ kinetically indistinguishable from $E_1$. Results in Figs. 4 and 5 allow to conclude that both superphosphorylation and the increase in phosphorylation
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rate (Figs. 1 and 2) could be reproduced by Scheme 1.
Simulations of Scheme 1 were also run using different initial proportions of E, with respect to E1. Increasing the proportion of E1 increased both the low affinity effect of ATP on \( r_m \), and superphosphorylation. However, if the fraction of E1 is sufficiently lowered superphosphorylation disappears, but the low affinity effect on velocity, albeit reduced, persists. These results were consistent with those of a series of experiments (not shown) in which the low affinity effect of ATP on the phosphorylation rate was tested on a Na,K-ATPase prepared by the procedure of Jensen et al. (1984) under optimal conditions (Esmann, 1988) and maximum steady-state level of phosphoenzyme. It was found that, with Jensen’s preparation, the function relating \( r_m \) to the concentration of ATP was similar to that in Fig. 2. Nevertheless, two main quantitative differences were detected, namely that (a) the value of the exponent \( n \) that was 2 for the experiment in Fig. 2, was near 3 with the less pure enzyme and (b) the ratio of \( r_m \) at 500 \( \mu \text{M} \) ATP to \( r_{m_{\text{max}}} \) was 1.8 for the experiment in Fig. 2 and 6.4 with the less pure preparation. Therefore the Na,K-ATPase prepared according to Jensen et al. (1984), showed not only a larger superphosphorylation level but also a larger low affinity effect of ATP on \( r_m \) than the zonal enzyme. This would be accounted for by Scheme 1, if in the zonal enzyme the relative concentration of E1 at the starting of the assay were smaller than in Jensen’s enzyme.

DISCUSSION
The main conclusion that can be drawn from the results presented in this report is that, at concentrations much higher than those needed for full saturation of its site in E1, ATP acted as a strong activator of the rate of phosphorylation. It is probable that this effect was not reported before because, to the best of our knowledge, none of the previous studies has employed concentrations of ATP as high as those used in the experiments described here. For example Mårdh and Zetterqvist (1974) examined the dependence on ATP of the apparent rate constant of phosphorylation of the Na,K-ATPase using at most 100 \( \mu \text{M} \) ATP. Although their findings agree very well with ours, within the range of concentrations they employed, for these concentrations the low affinity activation by ATP is not detectable (see Fig. 2).
In agreement with other authors (Campos and Beaugé, 1992; Rossi and Nørby, 1993), the time courses of phosphorylation at low [ATP] displayed a lag. Simulations showed that the lag disappeared when the rate constants of the formation of E1,ATP were sufficiently increased. This is to be expected from a reaction in which the formation of E1,ATP precedes phosphorylation and in which E1,ATP is not in equilibrium with its constituent species. Since the rate of binding of ATP rises linearly with the concentration of the nucleotide, increasing the concentration of ATP shifted the lag towards the origin, so that at concentrations higher than 200 \( \mu \text{M} \), the maximum rate of phosphorylation became indistinguishable from its initial rate.
If phosphorylation involved the binding of a single molecule of ATP per event, as postulated by the Albers-Post model, the low affinity parabolic effect on \( r_m \) cannot be explained. We reached the same conclusion for the case of superphosphorylation (Peluffo et al., 1992).

None of the curves showed the “heterogeneous” behavior of the Na,K-ATPase, reported by Mårdh and Post (1977) or either ATP or ADP (Klodos and Ottolenghi, 1985) accelerates phosphorylation. If this effect were the cause of the low affinity increase in \( r_m \) with ATP, a limiting reaction accelerated by high concentrations of ATP should exist, even in the presence of enough Na+ as to drive all the enzyme into E1 (Karlish and Yates, 1978; Skou and Esmann, 1983). If this reaction existed, under steady-state conditions ATP would increase both phosphoenzyme level and Na-ATPase activity with a \( K_0.5 \) much higher than the \( K_e \) of the Na-ATPase. Results in the literature show that this is not the case (Suzuki et al., 1987; Peluffo et al., 1992; Campos and Beaugé, 1992). Furthermore, the disappearance of the low affinity effect of ATP when the enzyme underwent turnover allows to discard actions of ATP on any of the steps that lead from E2P to E1. This lends support to the idea that the low affinity effect of ATP on \( r_m \) requires a form of the enzyme that is different from those present during the steady-state ATP hydrolysis by the Na,K-ATPase. A similar proposal has been made by us to account for superphosphorylation (Peluffo et al., 1992) which was also apparent in the experiments shown in this report.
For both the experimental and the simulated results, the estimated values of \( K_e \) (Equation 3) were 80–100 times higher than those reported for the equilibrium constant for the dissociation of ATP from its high affinity site (Nørby and Jensen, 1971; Jensen and Ottolenghi, 1983) and for the \( K_e \) for steady-state Na+ATPase activity (Rossi and Garrahan, 1985). In the case of the simulated results, this discrepancy is caused by the lack of rapid equilibrium binding of ATP, since if the values of the rate constants for binding and release of the nucleotide are increased by a factor of 1000, the simulated values for \( r_m \) for the high affinity part follow a Michaelis-Menten-like equation with \( K_m = 0.2 \mu \text{M} \). It is likely that non-equilibrium binding of ATP also holds for the experimental conditions. We have shown that this is necessary to account for the steady-state kinetics of the Na-ATPase (Rossi and Garrahan, 1985).

Taken together, results from the experiments and the simulations suggest that the low affinity effect of ATP on the rate of phosphorylation and superphosphorylation may both be expressions of a property of a conformer of the Na,K-ATPase that disappears as soon as the enzyme starts to undergo steady-state turnover. This is not predicted by the current versions of the Albers-Post model. Scheme 1, however, preserves the possibility of using this model to describe the steady-state behavior of the enzyme. It must be stressed that Scheme 1 was developed only to show that this is feasible. Whether it describes the reaction of the Na,K-ATPase remains an open question.

Acknowledgment—We thank Dr. Bliss Forbush III for permitting us to use the data processing program developed by him.
REFERENCES