Equilibrium of Phosphointermediates of Sodium and Potassium Ion Transport Adenosine Triphosphatase

Action of Sodium Ion and Hofmeister Effect

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ABSTRACT Sodium and potassium ion transport adenosine triphosphatase accepts and donates a phosphate group in the course of its reaction sequence. The phosphorylated enzyme has two principal reactive states, E1P and E2P. E1P is formed reversibly from ATP in the presence of Na^+ and is precursor to E2P, which equilibrates with P_i in the presence of K^+. We studied equilibrium between these states at 4°C and the effect of Na^+ on it. To optimize the reaction system we used a Hofmeister effect, replacing the usual anion, chloride, with a chaotropic anion, usually nitrate. We phosphorylated enzyme from canine kidney with [^{32}P]ATP. We estimated interconversion rate constants for the reaction E1P $\rightleftharpoons$ E2P and their ratio. To estimate rate constants we terminated phosphorylation and observed decay kinetics. We observed E1P or E2P selectively by adding K^+ or ADP respectively. K^+ dephosphorylates E2P leaving E1P as observable species; ADP dephosphorylates E1P leaving E2P as observable species. We fitted a 2-pool model comprising two reactive species or a twin 2-pool model, comprising a pair of independent 2-pool models, to the data and obtained interconversion and hydrolysis rate constants for each state. Replacing Na^+ with $Tris^+$ or $lysine^+$ did not change the ratio of interconversion rate constants between E1P and E2P. Thus Na^+ binds about equally strongly to E1P and E2P. This conclusion is consistent with a model of Pedemonte (1988. *J. Theor. Biol.* 134:165–182.). We found that Na^+ affected another equilibrium, that of transphosphorylation between ATP·dephosphoenzyme and ADP·E1P. We used the reactions and model of Pickart and Jencks (1982. *J. Biol. Chem.* 257:5319–5322.) to generate and fit data. Decreasing the concentration of Na^+ 10-fold shifted the equilibrium constant 10-fold favoring ADP·E1P over ATP·dephosphoenzyme. Thus Na^+ can dissociate from E1P·Na_3. Furthermore, we found two characteristics of Hofmeister effects on this enzyme.

KEY WORDS: Na • K • ATPase model

INTRODUCTION

The mechanism of metabolically driven active transport by a stoichiometric transport system, a P-type ATPase (Lutsenko and Kaplan, 1995; Lingrel and Kuntzweiler, 1994), includes a phosphorylated intermediate in the reaction sequence. In the case of sodium and potassium ion transport adenosine triphosphatase (Na,K-ATPase or the sodium pump)[1] this intermediate has two principal reactive states, which interconvert. Both reactive states are phosphorylated on the same aspartyl residue, Asp^{369} of the α-subunit (Cornelius, 1991; Jørgensen, 1992; Glynn, 1993; Robinson and Pratap, 1993; Horisberger, 1994). We studied equilibrium between these states.

Na,K-ATPase transports 3 Na^+ outward and 2 K^+ inward across the plasma membrane of most animal cells fueled by hydrolysis of the terminal phosphate group of 1 cytoplasmic ATP molecule, catalyzed by cytoplasmic Mg^{2+}. The principal states of the phosphointermediate are named E1P and E2P. E1P donates its phosphate group to ADP, forming ATP; this reaction is reversible and is stimulated by Na^+. E2P donates its phosphate group to H_2O, forming P_i; this reaction is reversible and is stimulated by K^+. In this paper E1P and E2P are defined only by the reactivity of the phosphate group, not by the binding of other ligands or by the sidedness of the transport site. A reader who attributes other meanings to E1P and E2P will find this article difficult to understand.

Taniguchi and Post (1975) synthesized ATP by converting E2P to E1P. First, they formed E2P from P_i and the free enzyme. Second, they added ADP and a high concentration of NaCl, 0.6 M. They recovered a little less than a stoichiometric amount of ATP. They attributed the synthesis to the high concentration of NaCl. In work presented here we estimated rate constants of interconversion between E1P and E2P and the effect of Na^+ upon them.

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[1]*Abbreviations used in this paper:* E1P, E2P, reactive states (forms, conformations) in the reaction sequence of Na,K-ATPase; Na,K-ATPase, sodium and potassium ion transport adenosine triphosphatase EC 3.6.1.37.

$$E1P \underset{k21}{\overset{k12}{\rightleftharpoons}} E2P; K_{eq} = k12/k21 . \quad (1)$$

K_{eq} is the ratio of the forward rate constant to the backward rate constant. We expected that increasing concentrations of Na^+ would drive the reaction backward and decrease the ratio, k12/k21, according to the following equation (Jørgensen, 1992; Horisberger, 1994):

$$Na_3E1P \rightleftharpoons Na_2E2P + Na^+. \quad (2)$$

We were surprised to find that the anion, Cl^-, was more effective than the cation, Na^+ in decreasing the ratio. Seeking a site of action of Na^+ we tested its effect on another reaction, that of transphosphorylation of E1P:

$$ATP \cdot E1 \rightleftharpoons ADP \cdot E1P; \; K_{int} = \frac{[ADP \cdot E1P]}{[ATP \cdot E1]} \quad (3)$$

Increasing concentrations of Na^+ decreased K_{int}, giving a preponderance of ATP·E1. Judging from the effects on the ratio of interconversion constants, we concluded that Na^+ combines more strongly with E1 than with E1P and about equally strongly with E1P and E2P, probably as follows:

$$Na_3E1 \rightleftharpoons Na_3E1P \rightleftharpoons Na_2E1P + Na^+ \rightleftharpoons Na_2E2P + Na^+. \quad (4)$$

We estimated interconversion rate constants (Eq. 1). To estimate rate constants accurately we needed to adjust the amounts of E1P and E2P. We controlled the partition between intermediates by varying the anion composition of the medium. Anions bias the partition between the phosphointermediates, E1P and E2P, according to a Hofmeister series (Post and Suzuki, 1991; Klodos, 1991; Klodos and Plesner, 1991). In a Hofmeister series, ions range from cosmotropic at one end to chaotropic at the other end (Collins and Washabaugh, 1985). Cosmotropic anions are carboxylate, sulfate, and phosphate, for instance. Chloride is intermediate. Bromide, nitrate, and iodide, thiocyanate or perchlorate are progressively more chaotropic. Cosmotropic anions favor E2P whereas chaotropic anions favor E1P (Post and Suzuki, 1991; Klodos, 1991).

MATERIALS AND METHODS

Rationale

Think of an isomerization between two species, A and B. At equilibrium the ratio of their concentrations is determined by an equilibrium constant, K_{ab}, as follows: $[B]/[A] = K_{ab}$. Imagine that a ligand, L, combines with B and not with A thus: $L + B \rightleftharpoons LB$. At equilibrium the ratio of their concentrations is determined by another equilibrium constant, K_{lb}, as follows: $[LB]/([B] \times [L]) = K_{lb}$. Suppose that experimentally we can not distinguish between B and LB so that we observe B + LB as a single species: $[B] = [A] \times K_{ab}$ and $[LB] = [B] \times [L] \times K_{lb}$. The ratio $([B] + [LB])/[A] = [B]/[A] + [LB]/[A] = K_{ab} + ([B] \times [L] \times K_{lb})/[A] = K_{ab} + (K_{ab} \times [L] \times K_{lb}) = K_{ab} \times \{1 + ([L] \times K_{lb})\}$. Thus the apparent equilibrium constant increases as [L] increases. Conversely, if there is no effect of ligand concentration on an apparent equilibrium, one can conclude that the ligand does not combine with one species more strongly than with the other. In designing these experiments we estimate apparent equilibrium between E1P and E2P and ask if Na^+ combines with E1P more strongly than with E2P. In the DISCUSSION, we explain why we do not detect a difference between forms of E1P combined with Na^+ and those free of Na^+. The reason is that Na^+ equilibrates with E1P more rapidly than our assay estimates the amount of E1P. Apart from the binding of ligands, the chemical composition of E1P is assumed to be the same as that of E2P.

Enzyme Preparation

Na,K-ATPase from the outer medulla of kidney was prepared by the step or continuous sucrose gradient methods of Jørgensen (1988). The dissection was modified as follows to improve the yield. Dog kidneys obtained fresh from another laboratory were stored at −70°C. On the day of preparation 10–12 kidneys were thawed in imidazole-sucrose solution (Jørgensen, 1988) at room temperature. Kidneys of poor quality were discarded. The capsule was removed and the kidney was sliced from one pole to the other; the plane of the cut passing through the hilus. A half kidney was placed, cut side down, on a glass surface cooled with ice. All the cortex was scraped off with a surgical scalpel starting at the edge and working toward the center. The blade of the scalpel was pressed lightly down and vigorously sideways to tear off the more friable cortex from the tough connective tissue surrounding and protecting the inner medulla.

The surface of the fibrous connective tissue is granular and its color is paler than that of the cortex. The scraped whole medulla was turned over and the inner medulla was removed by sharp dissection with curved scissors. The fibrous renal pelvis and its projections, the calices, were removed. The pale pyramids were excised. In places where pale inner medulla lay on top of outer medulla, the scissors were held open with the concave side upward. They were pressed down on the outer medulla until it bulged between the blades. The bulge was cut off. The yield was about 90 g of outer medulla from about 570 g of kidney. Ice cold imidazole-sucrose (160 ml) was added to the outer medullas in a cooled Waring blender, for which the voltage was reduced from 115 to 75 V. Homogenization in the Waring blender was done three times for 30 s each time. More homogenizing solution was added (340 ml), and homogenization was repeated once for 30 s. The homogenate was filtered through four layers of surgical gauze and was centrifuged for 15 min at 4,720 g_{avg}. The supernatants were transferred to fresh tubes and centrifuged for 45 min at twice the previous speed. The pellets were suspended in 240 ml of imidazole-sucrose and were centrifuged at 4,720 g_{avg} for 15 min. The yield of microsomes from this supernatant was 5.5 mg/g of outer medulla. The rest of the procedure was as described by Jørgensen (1988). The purified enzyme was stored at 8°C in glycerol 30% (wt/vol) dissolved in histidine 40 mM, H_4EDTA 1 mM (pH 7.2). Storage for 55 d did not change specific activity and phosphorylation capacity of one preparation but partition between E1P and E2P shifted in favor of E2P by about 6%. Estimation of E1P and E2P is described below. Light and dense fractions from a continuous gradient preparation showed 6% more E2P in the light fraction. The same preparation was used for control and test data except as noted since in four, possibly exceptional, cases we have seen much larger differences in E1P-E2P partition between different preparations of the enzyme. (See Fig. 3 in Post and Suzuki, 1991; other cases are not shown. See also Forbush, 1987.) The

range of specific activity in 20 step gradient preparations was 19–35 μmol P_i/min/mg at 37°C; the range of the highest specific activity in continuous gradient fractions was 27–46 μmol P_i/min/mg in 18 preparations (compare Jørgensen, 1994). Other procedures were as described previously (Vasallo and Post, 1986) except that the [^{32}P]phosphointermediate was estimated as described below.

Phosphointermediate

All experiments on the phosphointermediate were done at 4°C. The enzyme, ~2.4 U (μmol P_i/min), was phosphorylated from 0.01 mM [γ-^{32}P]ATP (0.01 μmol) in 40 mM imidazole MOPS (pH 7.1) and 0.10 mM $MgCl_2$ (0.10 μmol) as described previously (Vasallo and Post, 1986) with additional components given in the legends to the figures. The phosphorylation volume was 1 ml, and subsequent additions were in 0.1 ml, which contained salts at the same concentrations as those in the phosphorylation medium. This precaution avoided jumps in [salt], which produce unique effects (Klodos et al., 1994). To observe decay kinetics, phosphorylation was interrupted by an optimal chase solution containing a chelator of Mg^{2+}, 2 μmol 1,2-cyclohexylenedinitrilotetraacetic acid, and 0.1 μmol unlabeled ATP in 0.1 ml as described previously (Suzuki and Post, 1991). Additions to the stirred reaction mixture were made from a hand-held pipette at intervals timed by the sound of a metronome. At various times the labeled enzyme was denatured with 10 ml of 5.7% (wt/vol) trichloroacetic acid, 0.6 mM ATP, and 0.1% (wt/vol) H_3PO_4 or polyphosphoric acid. It was filtered, washed, and counted as described previously (Fukushima and Post, 1978). In all experiments reported here the ratio of initial amount of phosphoenzyme to protein was proportional to the specific activity of the enzyme preparation except in two cases noted in the text.

Phosphointermediate Components

These were estimated as follows. K^+-resistant E1P was isolated by including K^+ in the optimal chase solution and observing decay kinetics as described above for the absence of K^+. K^+ dephosphorylates E2P immediately (on the time scale of these experiments). ADP-resistant E2P was isolated by including ADP in the optimal chase solution and observing decay kinetics as described above in the absence of ADP. ADP dephosphorylates E1P immediately (on the time scale of these experiments). A chase is the time course of decay of phosphointermediate after formation of phosphoenzyme has been terminated by addition of a chase solution. A chase can also be the procedure that produces a chase. Chases without addition of ADP or K^+ are called "blank." Chases with addition of both ADP and K^+ were included in most experiments. Levels of phosphointermediate after these chases (<5%) were subtracted from levels at corresponding times after ADP-chases as a correction for an insufficient concentration of ADP since preliminary experiments showed that high concentrations of some salts reduced the affinity of the phosphoenzyme for ADP. The corrected levels are shown in the figures and were used in estimating kinetic constants. The concentrations of ADP and of K^+ are given in the figure legends.

Delayed Chases

To estimate rate constants accurately, delayed chases were included with standard chases in some experiments. Standard chases included addition of ADP or K^+ with the optimal chase solution (described under *Phosphointermediate*). A delayed chase was started with the optimal chase solution and ADP or K^+ was added in 0.1 ml after an interval of time. The amounts of ADP and K^+ are given in the legend to Fig. 1. After a second interval of time, acid was added to stop the reaction.

Kinetic Constants

The following 2-pool model was fitted to the data.

$$\begin{array}{lcl} & \overset{k12}{\rightleftharpoons} & \text{Interconversion Rate Constant} \\ E1P & & E2P \\ & \underset{k21}{\rightleftharpoons} & \text{Interconversion Rate Constant} \\ k10 \downarrow & & \downarrow k20 \quad \text{Hydrolysis Rate Constants} \\ E1 & & E2 \\ + & & + \\ P_i & & P_i \end{array} \qquad (5)$$

The analytical equation for the 2-pool model is given in (Klodos et al., 1981) with a different notation; our k12, k21, k10 and k20 are their k_2, k_{-2}, k'_{-1}, and k_3, respectively. Four rate constants, k12, k21, k10, and k20, and the value of E1P at zero time were five variable parameters fitted simultaneously to all the data points including those of the blank chases. The fitting program was "Don't-Use-Derivatives" from SAS Institute Inc., Cary, NC. The standard error in the tables and the values shown by the error bars in the figures are the asymptotic standard error reported by the program. The action of the program is described in the APPENDIX.

In a 2-pool model the decay curves for E1P and E2P are straight lines on a semi-logarithmic plot. In many experiments curved lines fitted the data better. To model curved lines we used a twin 2-pool model. The twin 2-pool model is the sum of two independent 2-pool sub-models.

One sub-model has rapid rate constants and the other has slow rate constants (see APPENDIX and Fig. 11). We used a twin 2-pool model since Suzuki and Post (1991) showed that our preparation of Na,K-ATPase can consist of separate rapid and slow varieties. Specifically they isolated the slow variety by decreasing the rate of phosphorylation of the enzyme from [γ-^{32}P]ATP to such a low level that only the slow variety accumulated. They decreased the rate of formation of the phosphointermediate by reducing the concentration of free Mg^{2+}. To isolate the rapid variety they phosphorylated with unlabeled ATP, stopped further phosphorylation with a chelator of Mg^{2+}, and let dephosphorylation proceed for a short time to accumulate the dephosphorylated form of the rapid variety. To this form they added [^{32}P]ATP and excess Mg^{2+} to label selectively the rapid variety. The rate constant of decay of the rapid variety was fivefold larger than that of the slow variety.

In the twin 2-pool model each rate constant in the rapid submodel is larger than the corresponding rate constant in the slow sub-model by a single factor. Thus the ratio of the rate constants is one of two parameters added to the plain 2-pool model. The other additional parameter is the ratio of the amounts of the submodels. In experiments with insufficient data points to determine the additional parameters precisely, the ratio of amounts was set to 1 arbitrarily. Thus the twin 2-pool model has only 1 or 2 more parameters than the plain 2-pool model. The way to set up the computer program, including the equations, is outlined in the APPENDIX.

Pickart-Jencks Experiments

Phosphorylation was performed as above except that 0.1 mg of bovine serum albumin was included, Mg was present as 0.5 mM $Mg(NO_3)_2$, and the salt was 0.5 M $NaNO_3$. The presence of this

salt increased the amount of E1P. In some experiments arginine$^+$, *N*-methylglucamine$^+$, or triethylamine$^+$ replaced some of the Na^+ as indicated in Table III. The counterion remained nitrate. The labeled phosphoenzyme was chased with a final concentration of 6 mM KCl and sufficient unlabeled ATP or a chelator of Mg^{2+} (for blank chases) to prevent further formation of the labeled phosphointermediate. Concentrations of K^+ between 6 and 100 mM all gave the same amount and rate of decay of phosphointermediate (not shown). This procedure gave ~90% of the phosphointermediate as K-resistant E1P. For ADP chases of E1P the concentration of free Mg^{2+} was kept constant. Free Mg^{2+} in the chase mixture decreased the effect of ADP even in amounts that did not form a significant amount of MgADP complex. A concentration of free Mg^{2+} greater than 0.3 mM saturated this effect. Free [Mg^{2+}] and free [ADP] were calculated from a computer program in which dissociation constants were 0.0603 mM for MgATP and 0.676 mM for MgADP and were 105 mM for NaATP and 224 mM for NaADP (Smith and Martell [1975] give more accurate values).

ATPase Activity

The reaction mixture contained 40 μmol imidazole MOPS, 1 μmol Na_3EGTA, salts to give the concentrations indicated in the legends, and the enzyme in 0.9 ml. In each reaction mixture the salts had only one anion as indicated. After 20 min at 37°C the reaction was started by addition of 0.1 ml containing 4 μmol of ATP (4 mM final concentration). After another 20 min the reaction was stopped by addition of 0.5 ml of 5% (wt/vol) sodium dodecylsulfate. Inorganic phosphate was estimated by automated analysis (Hegyvary et al., 1979). Data were not taken at intermediate times. The rate was the difference between the amount of inorganic phosphate after the reaction was stopped minus the amount of inorganic phosphate immediately after the addition of ATP divided by the duration of incubation, 20 min.

RESULTS

Outline of Procedure

The equilibrium constant between phosphointermediates E1P and E2P was estimated from the ratio of forward to backward interconversion rate constants (Eq. 1). Rate constants were estimated from chase experiments as follows. Phosphointermediates were formed from [^{32}P]ATP, usually reaching a steady state of formation and breakdown before analysis. Formation was interrupted by addition of the optimal chase solution and decay kinetics were observed as described under *Phosphointermediate Components* in MATERIALS AND METHODS. Decay kinetics after a blank chase (without K^+ and ADP) were those of E1P and E2P together. K^+-resistant E1P and ADP-resistant E2P were estimated separately by adding K^+ or ADP to the optimal chase solution. Decay kinetics of E1P or E2P or of both together (after a blank chase without K^+ and ADP) were observed separately and the combined data were fitted to a model simultaneously. The model includes both interconversion and hydrolysis rate constants to accommodate breakdown of the intermediates. The principal experimental variables were the concentration of Na^+ or the anion composition of the medium. Specific results are given later.

A 2-pool Model

To estimate rate constants a model is necessary. We used the 2-pool model and equations of Klodos et al. (1981) to estimate rate constants of interconversion between E1P and E2P. The 2-pool model is described under *Kinetic Constants* in MATERIALS AND METHODS (Eq. 5).

Operation of the 2-pool Model

In estimating an equilibrium one ordinarily allows the components of a system to react until the composition is constant in time. However, in the case of the phosphointermediates of Na,K-ATPase, equilibrium is not reached because hydrolysis is significant. Instead a steady state of formation and breakdown is reached. In this state hydrolysis of E2P reduces the amount of E2P and shifts the partition between E1P and E2P in favor of E1P. Thus the steady state partition is not the equilibrium partition. "Partition" is used here to mean a division into parts, specifically division of the phosphointermediate into a pool of E1P and a pool of E2P.

In our experiments, rate constants of Eq. 5 were estimated from decay kinetics of the phosphointermediates following chases that left E1P, E2P or both as the observed species. The rate constant of decay of E1P is the sum of k12 and k10. The rate constant of decay of E2P is the sum of k21 and k20. The decay of the combined intermediates is the sum of hydrolysis of E1P and E2P. Rate constants k12 and k21 are interconversion rate constants; k10 and k20 are hydrolysis rate constants. The equations of Klodos et al. (1981) predict these decays and allow a statistical program to estimate rate constants that fit a set of data. Given k12 and k21 the equilibrium constant is their ratio, k12/k21 (Eq. 5).

Criterion of a Steady State in the Model

The phosphointermediate can be tested for a steady state of formation and breakdown by the behavior of one of its components, E2P, after termination of formation. After termination of formation the phosphointermediate components decay. E1P always decreases progressively in amount with time since it is the precursor form. In contrast, the amount of E2P does not change immediately after termination of formation; it is still being formed by conversion of E1P to E2P. E2P decays secondarily to the decay of E1P. In these experiments the behavior of E2P after termination of formation was examined by reference to the model fitted to the data. The behavior of E2P after termination of formation by a blank optimal chase solution, without addition of ADP or K^+, is shown by dashed lines in the lower panels of Figs. 1–3, *E2P*. If the rate of change of the amount of E2P with time, extrapolated back to zero time, is zero, the phosphointermediate was in a steady state of formation and breakdown just before formation was termi-

nated. Conversely finding a zero rate of disappearance of E2P in experiments in which a steady state is expected from other criteria supports use of the model.

A Twin 2-pool Model

For data where the 2-pool model did not fit, due to nonlinearity of decay data on semilogarithmic plots, we expanded the 2-pool model to a twin 2-pool model (see Fig. 11). The twin 2-pool model is the sum of a pair of independent 2-pool submodels. Each rate constant of one submodel is larger than the corresponding rate constant of the other submodel by a single factor. Thus the submodel with the more rapid rate constants can be called "rapid" and the submodel with the slower rate constants can be called "slow." In addition to this factor, the ratio of rate constants, there is another parameter. This parameter is the relative amounts of the phosphointermediate of the two submodels in the initial amount of phosphointermediate, before addition of the chase solutions. Thus the twin 2-pool model has only two more parameters than the plain 2-pool model, namely the ratio of the rate constants of the submodels and the ratio of the initial amounts of phosphointermediate in the submodels. There are four pools of phosphointermediate in all. Two of these pools are E1P and E2P in a rapid submodel with rapid rate constants and the other two are E1P and E2P in a separate slow submodel with slow rate constants. Observed E1P is the sum of E1P in the rapid submodel plus E1P in the slow submodel. Observed E2P is the sum of E2P in the rapid submodel plus E2P in the slow submodel. The equation of each of the submodels is Eq. 5.

In cases where the data were not sufficient to specify the relative amounts of submodels precisely, we arbitrarily set the amounts to 50% for each submodel. See references (Klodos et al., 1994; Post and Klodos, 1996) for discussion of functional heterogeneity of this enzyme.

Validity of the 2-pool or Twin 2-pool Model

Since there are many models of the phosphointermediate in the literature, particularly ones including an intermediate between E1P and E2P in the reaction sequence (Robinson and Pratap, 1993), it was important to test the suitability of the 2-pool model to represent data. To make the test stringent we introduced delayed chases (see also Froehlich and Fendler, 1991). In this technique formation of labeled phosphointermediate (E1P plus E2P) is terminated by addition of the optimal chase solution containing unlabeled ATP (or a chelator of Mg^{2+} or both). An interval of time follows this addition before further addition of a ligand, K^+ or ADP, to estimate K-resistant E1P or ADP-resistant E2P respectively. Thus interconversion between E1P and E2P proceeds for an interval in the absence of K^+ and ADP. This interval permits rate constants in the absence of K^+ and ADP to be tested for consistency with rate constants in their presence.

To test the model an experiment was conducted in 150 mM NaSCN in place of the usual NaCl. Thiocyanate favors E1P in its action on the processes that partition the phosphointermediate between E1P and E2P (Post and Suzuki, 1991). Fitting the twin 2-pool model to the data (Fig. 1) gave a good fit (Fig. 1, *solid lines*) and well-defined rate constants (Table I). (With the plain 2-pool model the residual error was fourfold larger than with the twin 2-pool model.) For both E1P (Fig. 1, *top*) and E2P (Fig. 1, *bottom*) there was a higher level of phosphointermediate after a delayed chase (□, △) than after a standard chase (■, ▲) at the same time values. These increases indicate that interconversion took place in both directions during the delay interval. The only source for either form during the delay was its partner, the other form. For an example consider E1P. The logic is the same for E1P or E2P. Consider what happens after K^+ is added at zero time; all E2P is dephosphorylated and any E2P that is formed later from E1P is dephosphorylated. E1P decays all by itself. It decays by conversion to E2P (k12) and by hydrolysis (k10). Compare that situation with the situation during a blank chase after addition of the optimal chase solution without addition of K^+. No further formation of E1P from ATP is taking place. E1P is being converted to E2P and is hydrolyzing as before. But in this case E2P is still present. E2P is being converted back to E1P at some rate during the interval of the delay. After the delay period K^+ is added and all E2P is dephosphorylated; only E1P remains. And during its subsequent decay at each time point there is more E1P than there was at the same time point when K^+ was added at zero time. Where did this extra E1P come from? It could not have come from ATP since all formation from ATP was stopped at zero time. It came from the E2P that remained during the delay period. The same logic applies correspondingly to the extra E2P that remained after a delayed ADP chase. Since the extra phosphointermediate was larger for E2P than for E1P, there was more conversion from E1P to E2P than from E2P to E1P. The consistency of the data with the model indicates that K^+ and ADP had little effect on the rate constants. That is, k10 and k12 were not affected by K^+ and k20 and k21 were not affected by ADP (Eq. 5). The rate constants were the same during the blank chase in the absence of K^+ and ADP as they were during the K^+- and ADP-chases.

The fitted model, with parameters from Table I, was used to calculate the behavior of E1P and E2P in the absence of K^+ and ADP (Fig. 1, *dashed lines*). The dashed lines show the calculated time course of disappearance of E1P and E2P in the absence of K^+ and

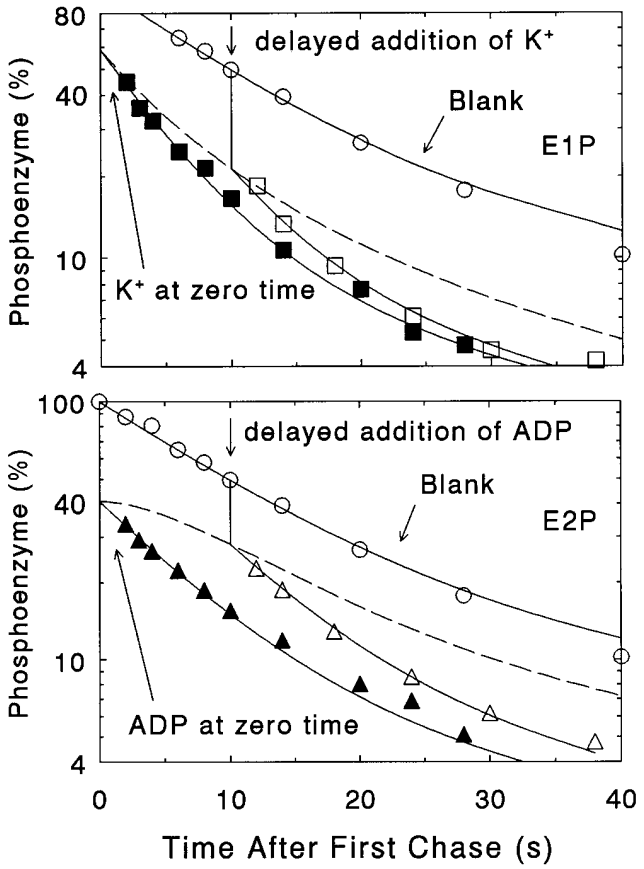

FIGURE 1. Fit of a twin 2-pool model to standard and delayed K- or ADP-chases. The medium contained 150 mM NaSCN to obtain a high proportion of E1P in this preparation. (Na,K)-ATPase was phosphorylated from ATP for 40 s as described under *Phosphointermediate* in MATERIALS AND METHODS. This period of phosphorylation allowed partition of the phosphointermediate between E1P and E2P to reach a steady state. At zero time further formation of the phosphointermediate was terminated by addition of the optimal chase solution described under *Phosphointermediate* in MATERIALS AND METHODS. After various intervals of time acid was added to stop the reaction and the amount of phosphointermediate was estimated. These amounts were the sum of E1P and E2P showing the kinetics of its disappearance during a blank chase (○) in the absence of K^+ and ADP. E1P and E2P were estimated separately as described under *Phosphointermediate Components* in MATERIALS AND METHODS. K-resistant E1P was estimated by addition of KCl to the optimal chase solution to give a final concentration of 6 mM KCl (■ or □, *E1P*) at zero time (■) as a standard chase or 10 s later (□) as a delayed chase (*top*) as described under *Delayed Chases* in MATERIALS AND METHODS. ADP-resistant E2P was estimated by addition of ADP to the optimal chase solution to give a final concentration of 0.84 mM ADP (▲ or △, *E2P*) in corresponding standard (▲) or delayed (△) chases (*bottom*). A twin 2-pool model was fitted to the data. For a description of the model see the APPENDIX. The kinetic constants are in Table I. The solid lines show values in the model. E1P is shown in the upper panel and E2P is shown in the lower panel. The dashed lines show the amounts of E1P (*top*) and E2P (*bottom*) in the model during the blank chase in the absence of K^+ and ADP. For the blank chase the data (○) and fitted lines are the same in both panels.

ADP according to the model. The slope of the calculated amount of E2P against time at the start of the blank chase was close to zero (Fig. 1, *lower panel dashed line*, and Table I). This result is consistent with a steady state of the phosphointermediates at 40 s after the start of phosphorylation from ATP before addition of the optimal chase solution. The mechanism is explained under *Criterion of a Steady State in the Model*. The method of calculation is indicated in the APPENDIX.

We repeated this test of a 2-pool model in 240 mM nitrate replacing 210 mM of the usual Na^+ with $Tris^+$ in order to decrease all rate constants. (The stimulatory effect of Na^+ is shown later in Figs. 4 and 5.) The duration of phosphorylation before the chase was 30 s, delayed chases were started at various intervals after a standard chase, and all were terminated with acid 4 s after addition of K^+ or ADP.

Again the twin 2-pool model fitted the data (Fig. 2, *solid lines*) and gave well-defined rate constants (Table II). Again the amount of E2P versus time in the absence of K^+ and ADP was calculated from the parameters of the model (Fig. 2, *lower panel dashed line*). Again the slope of the calculated amount of E2P at the start of the chase was close to zero, indicating that E1P and E2P were in a steady state just before the chases began and that 30 s of phosphorylation was sufficient time to develop a steady state.

To test the model in a state preliminary to a steady state, we repeated the experiment with a shorter interval of phosphorylation, namely 6 s. Again a good fit to the data (Fig. 3, *solid lines*) and rate constants (Table II) were obtained. The precursor–product relationship of E1P to E2P implies that the partition between them is in favor of E1P before a steady state is reached. This result was found. The time course of the amount of E2P

TABLE I

Parameters of Twin 2-pool Model Fitted to Delayed Chases in 150 mM NaSCN (from Fig. 1)

Parameter	Units	Value*	SEM
Hydrolysis of E1P, k_{10}	/s	0.077	0.009
Hydrolysis of E2P, k_{20}	/s	0.072	0.009
Conversion of E1P to E2P, k_{12}	/s	0.075	0.008
Conversion of E2P to E1P, k_{21}	/s	0.038	0.005
Equilibrium constant, $K_{eq} = k_{12}/k_{21}$		2.0	0.35
Amount of E1P at 0 time	%	59	0.76
d(E2P)/dt at 0 time	%/s	−0.1	
Residual mean square error	% EP	1.1	
Comparison of rapid & slow twins			
ratio of rates		6.3	2.1
amount of slow twin	%	16	4.6

Experimental procedure is in the legend to Fig. 1. *The APPENDIX shows how this value was calculated.

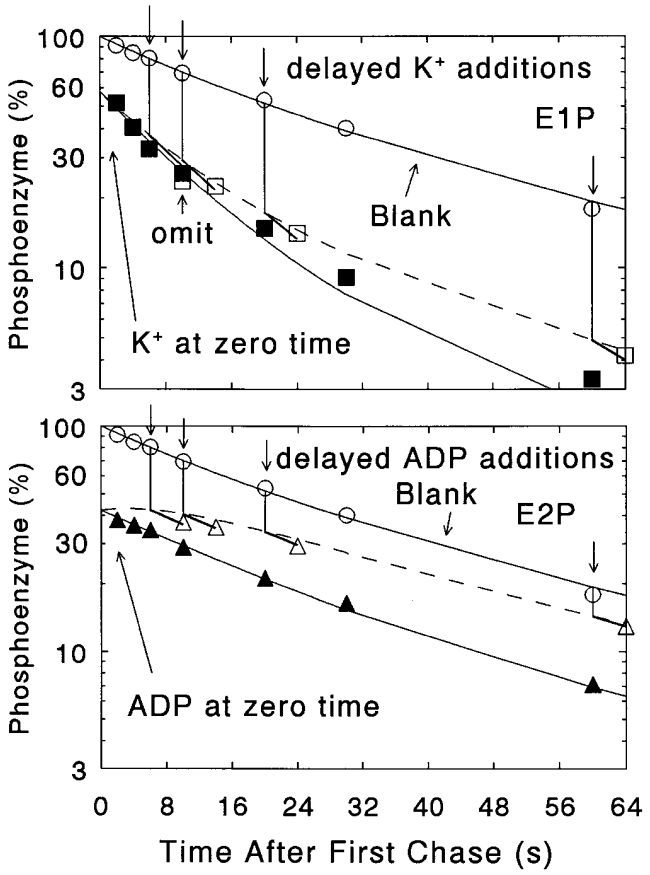

FIGURE 2. Fit of a twin 2-pool model to standard and delayed K- or ADP-chases after phosphointermediates reached a steady state. The experiments were the same as those in Fig. 1 except for the following differences. The enzyme preparation was different. A combination of 30 mM $NaNO_3$ plus 210 mM $TrisNO_3$ replaced NaSCN. The duration of phosphorylation was 30 s. The [ADP] was 0.57 mM. The interval between the blank chase and the delayed chases varied. For each delayed chase, shown by open squares or triangles, the addition of K^+ or ADP was 4 s before termination of the reaction by addition of acid. The times of addition of K^+ or ADP are show by arrows (↓). The interval of 4 s is shown by a short solid line preceding each of these data points. The line shows the time course of decay of E1P or E2P respectively in the model fitted to the data. The kinetic constants are in Table II. "Omit" indicates a data point not used in fitting the model. The symbols of the data points are the same as in Fig. 1: (○) blank chase, (■, □) K-resistant E1P, (▲, △) ADP-resistant E2P, (■, ▲) standard, or (□, △) delayed chases. The dashed lines show the calculated amounts of E1P (*top*) and E2P (*bottom*) in the model during the blank chase in the absence of K^+ and ADP.

in the absence of K^+ and ADP was calculated by inserting the fitted parameters (Table II) in the equations of the model (APPENDIX). The slope of the calculated amount of E2P plotted against time should be positive before a steady state is reached. The slope of the calculated amount of E2P was positive (Fig. 3, *lower panel dashed line*). An unexpected result was more rapid interconversion rate constants in the pre-steady state period (Table II). Rossi and Nørby (1993) found more rapid

TABLE II

Parameters of Twin 2-pool Model

Parameter	Units	Duration, s 6	30	*P**, %
Hydrolysis of E1P, k_{10}	/s	0.043	0.049	> 10
Hydrolysis of E2P, k_{20}	/s	0.024	0.025	> 10
Conversion of E1P to E2P, k_{12}	/s	0.052	0.036	0.1
Conversion of E2P to E1P, k_{21}	/s	0.021	0.012	2.3
Equilibrium constant, $K_{eq} = k_{12}/k_{21}$		2.5	3.0	
Amount of E1P at 0 time	%	72	58	> 0.1
d(E2P)/dt at 0 time	%/s	2.5	0.5	

Effect of duration of phosphorylation in Tris nitrate (from Figs. 2 and 3). Experimental procedure is in the figure legends. *Probability that the values at 6 and 30 s come from a single population.

rate constants in the first turnover of Na,K-ATPase than in subsequent turnovers.

The twin 2-pool model fitted the data well in experiments with long and short times of formation of phosphointermediates (Figs. 2 and 3). The analysis is consistent with a precursor–product relationship between E1P and E2P before the chases began. This relationship was shown earlier by a different method (Fukushima and Nakao, 1981).

Action of Na^+ on Rate Constants

To investigate the action of Na^+, experiments like those in Fig. 2 were repeated in different salt solutions. In one case the experiment in Fig. 2 was compared with another experiment in which $Tris^+$ was replaced with Na^+. The experiment in Fig. 2 was conducted in 30 mM $NaNO_3$ plus 210 mM Tris NO_3. This experiment was compared with a similar one conducted in 240 mM $NaNO_3$ (not shown). In 240 mM Na^+ the proportion of E1P and both hydrolysis rate constants, k10 and k20, were higher than in 30 mM Na^+. The interconversion rate constants were not affected significantly (Fig. 4). The rate constants are small because the temperature is 4°C. Thus Na^+ increased the proportion of E1P by stimulating hydrolysis of E2P and not by changing significantly the equilibrium constant of interconversion. (Hydrolysis of E1P increases the ratio of dephospho- to phospho-intermediates but in a steady state it does not affect the partition between E1P and E2P since the amount of E1P is constant in a steady state and E1P is the only precursor to E2P.) This pair of experiments was repeated with a different enzyme preparation. In this case the salt was 280 mM $NaNO_3$ and 250 mM of the Na^+ was replaced by $lysine^+$. The results were similar (not shown).

In the literature there is an experiment on Na,K-ATPase from ox brain in which the phosphointermediate was chased with K^+ or ADP in various concentra-

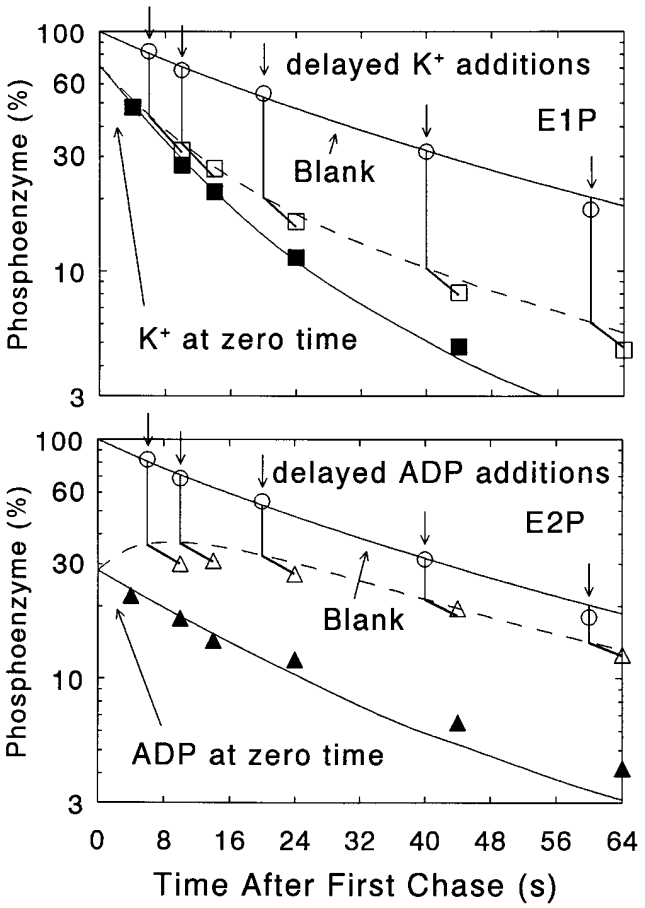

FIGURE 3. Fit of a twin 2-pool model to standard and delayed ADP- or K-chases before phosphointermediates reached a steady state. The experiments were the same with the same symbols as the corresponding experiments in Fig. 2 except that the duration of phosphorylation before the standard chase was 6 s instead of 30 s. The kinetic constants are in Table II.

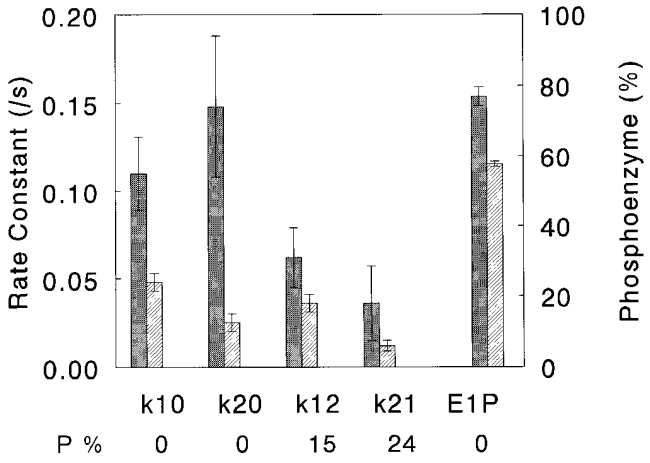

FIGURE 4. Effect of $[Na^+]$ on rate constants and initial amount of E1P. The experiment in Fig. 2 was repeated with substitution of Na^+ for $Tris^+$ and with a different fraction (from a continuous sucrose gradient) of the same enzyme preparation. A twin 2-pool model was fitted to the data of this experiment (see *Kinetic Constants* in MATERIALS AND METHODS and the APPENDIX for a description of the model). The model is the basis for the estimation of rate constants. The values of rate constants (Eq. 5) and amount of E1P from the experiment in 240 mM $NaNO_3$ are shown by dark bars, and those from the experiment in 210 mM $TrisNO_3$ plus 30 mM $NaNO_3$ (Fig. 2, Table II) are shown by light bars. The error bars show the asymptotic standard error calculated by the fitting program. *P* % shows the probability that the difference due to substitution of Na^+ for $Tris^+$ can be attributed to random variation in a single population of experiments.

tions of Na^+ at a constant Cl^- concentration (Nørby et al., 1983). Na^+ was replaced by $Tris^+$. To obtain rate constants we fitted a twin 2-pool model to these data. As $[Na^+]$ increased from 20 to 300 mM, E1P increased from 37 to 52% (not shown), and three rate constants (k12, k21, and k20) increased (Fig. 5). In this case also, Na^+ increased the proportion of E1P by stimulating (weakly) hydrolysis of E2P. Stimulation of both interconversion rate constants suggests a nonspecific action of Na^+. There was no significant hydrolysis of E1P, ($k10 \approx 0$), in contrast to the analysis of data from dog kidney in Fig. 4 and its companion experiment with $lysine^+$ in place of $Tris^+$ (not shown). We fitted a 2-pool or twin 2-pool model to data from the same laboratory on Na,K-ATPase from ox or pig kidney at various concentrations of NaCl in Fig. 3 in (Klodos and Nørby, 1987) and again found no significant hydrolysis of E1P. Thus the difference in hydrolysis of E1P may depend on the way in which the experiments are done or on the difference in species. Steinberg and Karlish (1989) found a functional difference in species between dog and pig kidney Na,K-ATPase.

Action of Na^+ on Hofmeister Effects

In these experiments we used Hofmeister effects to adjust the experimental system. Previous demonstrations of Hofmeister effects were made at high Na^+ concentrations. In chase experiments like those reported here, but simpler, the anion composition of the medium was varied. The ratio of the amount of E1P to the amount of E2P increased the more chaotropic the anion (Post and Suzuki, 1991; Klodos, 1991; Klodos and Plesner, 1991). To determine if a high $[Na^+]$ is needed to enable a Hofmeister effect we compared nitrate with acetate at 30 mM Na^+ with 250 mM Lys^+ as companion cation. The amount of E1P was sevenfold larger in nitrate than in acetate (Fig. 6). Thus nitrate was much more effective in stabilizing E1P than acetate was and Hofmeister anion effects were independent of a high concentration of Na^+. Since acetyl phosphate is a substrate for phosphorylation of Na,K-ATPase, the product, acetate, can react with E1P and deplete it. However, at 5 mM it does not (Beaugé et al., 1985) and at 400 mM it affects only 10% of the phosphointermediate (Taniguchi et al., 1988). Thus acetate probably did not dephosphory-

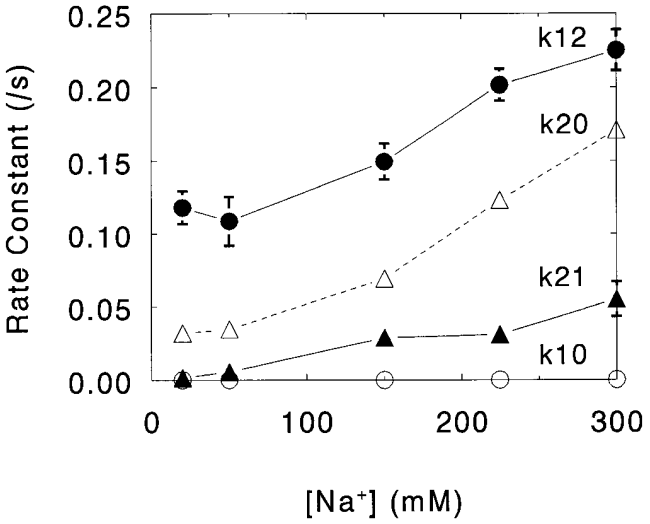

FIGURE 5. Effect of Na^+ on rate constants in the 2-pool model. The data are from experiments in Fig. 3 from (Nørby et al., 1983) plus one unpublished experiment at 50 mM Na^+ performed at the same time. The enzyme preparation was from ox brain and the medium was 300 mM NaCl with replacement of Na^+ by $Tris^+$. The twin 2-pool model was fitted to data values provided by Dr. Klodos. To reduce the number of variable parameters (and thereby increase the precision of the estimates), k10 was set equal to 0 since it was not significantly different from 0 when it was a variable. The mean square residual was <2.7% except at 50 mM Na^+, where it was 5.1%. The initial steady state value of E1P increased progressively from 37% at 20 mM Na^+ to 53% at 300 mM Na^+. k12 (●), k21 (▲), k10 (○), k20 (△). The ratio of k12/k21 is not significantly different from 5 at all concentrations of Na^+.

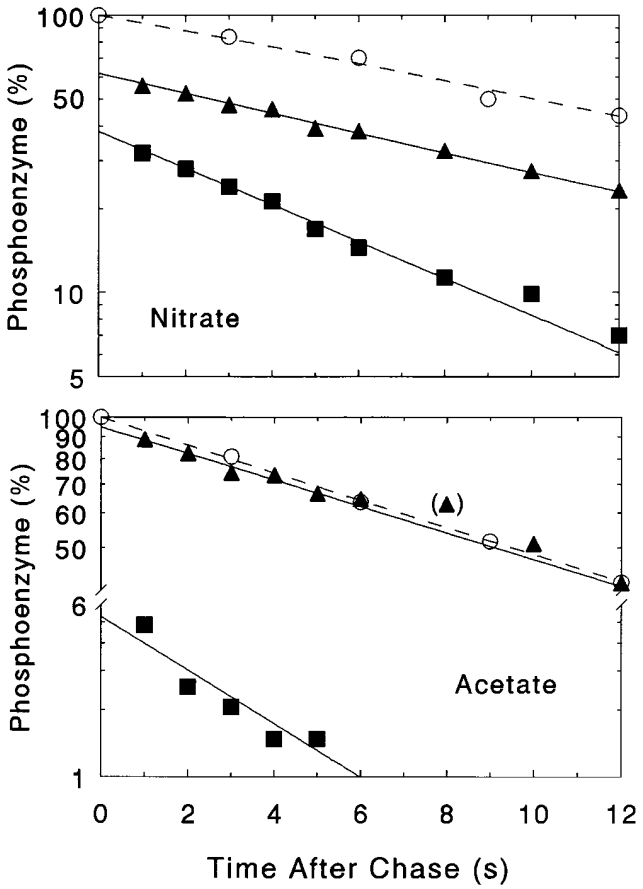

FIGURE 6. Effect of anions on partition of phosphoenzyme between E1P and E2P at low $[Na^+]$. The companion experiment to that in Fig. 4 (see text) was repeated with 280 mM nitrate or acetate as indicated. $[Na^+]$ was 30 mM and $[lysine^+]$ was 250 mM. The enzyme preparation was different from that in Fig. 4 and delayed chases were omitted. The symbols are the same as in Fig. 1. The lines fit a 2-pool model. Rate constants of the model were imprecise in acetate due to the small amount of E1P. The initial amount of E1P was 36% in nitrate and 5% in acetate. The data point in parentheses () was not used in fitting the model.

late E1P significantly. (In this experiment the ratio of initial amount of phosphoenzyme to protein was 25% lower than usual in acetate. We do not know a reason for this result. In another experiment with 200 mM acetate the ratio was the usual value.)

Effect of Anions on Interconversion Equilibrium

Post and Suzuki (1991) observed that replacement of a cosmotropic anion with a chaotropic anion increased the proportion of E1P and also decreased the rate of hydrolysis as indicated by the rate of the blank chase (their Fig. 5). A decrease in the rate of hydrolysis can not increase the proportion of E1P (*Operation of the 2-pool Model* in RESULTS). Thus we expected that replacement of a cosmotropic anion with a chaotropic anion shifts the interconversion equilibrium between E1P and E2P in favor of E1P. We compared experiments done in 160 mM NaCl, 150 mM NaSCN, 600 mM NaCl, and 200 mM Na_3citrate. These experiments were done on different enzyme preparations. We fitted a twin 2-pool model to each experiment and estimated the ratio of interconversion rate constants, k12/k21. The smaller this ratio the more E1P is favored in the equilibrium. In 160 mM NaCl the ratio was larger than 5 whereas in 150 mM NaSCN the ratio was 1.6. Thus replacement of chloride with thiocyanate shifted the equilibrium in favor of E1P. In 200 mM Na_3citrate the ratio was 4 whereas in 600 mM NaCl it was 0.25. Thus replacement of citrate with chloride also shifted the equilibrium in favor of E1P. (In the experiment in citrate the ratio of the initial amount of phosphoenzyme to protein was unusually low for no apparent reason.) Thus anions shifted the equilibrium of interconversion according to a Hofmeister series. Chaotropic ions favored E1P and cosmotropic anions favored E2P. Hydrolysis rate constants, k10 and k20, were lower the more chaotropic the anion. (Compare Fig. 7 in Post and Suzuki, 1991.)

Effect of Na^+ on Transphosphorylation from E1P to ADP

E1P donates its phosphate group reversibly to ADP forming ATP·E1. This step is the reverse of the forward reaction sequence. To investigate the action of Na^+ on

this step we adapted the procedure and model of Pickart and Jencks (1982), who used the calcium-ATPase of sarcoplasmic reticulum. Their procedure was to form the phosphointermediate from [γ-^{32}P]ATP under conditions where E1P was predominant and relatively stable. Then they interrupted formation with excess unlabeled ATP and observed decay kinetics in the presence of various concentrations of ADP. They assumed rapid equilibration of ADP binding and transphosphorylation from the phosphointermediate to form tightly bound ATP. These rapid steps were followed by slow dissociation of the bound ATP. Their model included an equilibrium constant for transphosphorylation as well as a dissociation constant for ADP and a rate constant for dissociation of ATP, which was rate-limiting. The adapted model is the following:

$$\mathrm{ADP} + \mathrm{E1P} \overset{K_S}{\rightleftharpoons} \mathrm{ADP \cdot E1P} \overset{K_{int}}{\rightleftharpoons} \mathrm{ATP \cdot E1} \overset{k_{off}}{\rightarrow} \mathrm{E1} + \mathrm{ATP}. \quad (6)$$

There are two equilibrium constants, K_S and K_{int}, whose reactions are rapid on the time scale of these experiments. There is one slow rate constant of irreversible dissociation, k_{off}. $K_S = [\mathrm{ADP}][\mathrm{E1P}]/[\mathrm{ADP \cdot E1P}]$. $K_{int} = [\mathrm{ADP \cdot E1P}]/[\mathrm{ATP \cdot E1}]$. $k_{off} = -\mathrm{d}(\mathrm{ATP \cdot E1})/[\mathrm{ATP \cdot E1}]/\mathrm{d}t$. The model predicts a biphasic dephosphorylation of E1P on a semilogarithmic plot. An instantaneous rapid phase is followed by a linear slow phase. The maximum extent of the rapid phase depends on K_{int}: ($\mathrm{extent}_{max} = 1/[1 + K_{int}]$) expressed as a fraction of the initial amount of E1P. (Note that K_{int} is defined with respect to formation of ADP·E1P as the forward direction.) The extent of the rapid phase depends on a saturation function of [ADP]: ($\mathrm{extent} = 1/\{1 + K_{int}(1 + K_S/[\mathrm{ADP}])\}$). For the slow phase the rate constant is the product of k_{off} and the extent. Thus the negative slope of the slow phase is proportional to the extent of the rapid phase.

We adjusted the reaction system as follows. To start with a large amount of E1P we used a Hofmeister effect and conducted the experiments in 500 mM $NaNO_3$. We formed the phosphointermediate from [γ-^{32}P]ATP and chased it with excess unlabeled ATP to prevent further formation of the radioactive intermediate. To isolate K-resistant E1P we included 6 mM KCl in the chase. To estimate the effect of ADP we included various concentrations of ADP in the chase. For the blank chase in the absence of ADP we replaced ATP with a chelator of Mg^{2+}, which is required for phosphorylation. We did this to avoid traces of ADP that might contaminate our source of ATP. To estimate the initial amount of E1P and its rate of hydrolysis and conversion to E2P we extrapolated the blank chase to zero time and used the rate constant of the blank chase as a baseline to be subtracted from the rate constant of each ADP chase to estimate the action of ADP.

The Pickart-Jencks model fitted the data well (Fig. 7). The lines drawn according to the model are close to the data points. Addition of ADP produced an immediate loss of intermediate, within 1 s, followed by a slow exponential decay (linear on a semilogarithmic plot). The immediate loss was due to equilibration of ADP with the phosphointermediate: ADP + E1P $\rightleftharpoons$ ADP·E1P $\rightleftharpoons$ ATP·E1 whereas the slow decay was due to slow dissociation of ATP: ATP·E1 $\rightarrow$ E1 + ATP. The rate constant of the slow decay was proportional to the immediate loss. To study the effect of Na^+ we replaced it with arginine$^+$, *N*-methylglucamine$^+$, or triethylamine$^+$ (Table III). k_{off} varied over a range, 0.7–1.9/s, without correlating with [Na^+]. (Compare 1–4/s in Klodos and Nørby, 1987). K_S for ADP varied over a range, 1–6 mM, without correlating with [Na^+]. K_{int} was 0.1–0.3 in 500 mM Na^+, was 0.5–1.4 in 200 mM Na^+ and was 2.1–2.5-fold higher in 45 mM Na^+ in each medium. K_{int} = [ADP·E1P]/[ATP·E1]. Thus decreasing concentrations of Na^+ shifted the transphosphorylation equilibrium constant to favor ADP·E1P.

Additivity of Hofmeister Effects

Hofmeister effects "are approximately additive over all species in solution" (Collins and Washabaugh, 1985).

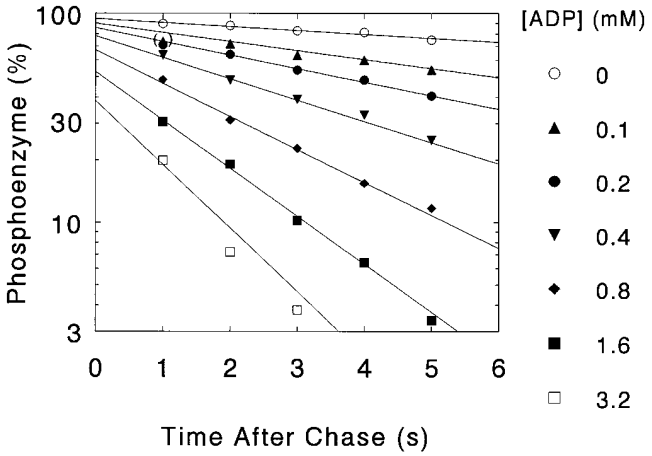

FIGURE 7. Effect of [ADP] on dephosphorylation of E1P fitted to a Pickart-Jencks model. The phosphointermediate was formed from [γ-^{32}P]ATP as described in MATERIALS AND METHODS with 500 mM $NaNO_3$ and 0.5 mM $Mg(NO_3)_2$ in place of other salts. After 20 s the labeled phosphoenzyme was chased with final concentrations of 6 mM KCl and 4.55 mM $(Tris)_4$1,2-cyclohexylenedinitrilotetraacetate for blank chases or with 6 mM KCl and 1 mM unlabeled ATP plus sufficient $Mg(NO_3)_2$ to keep the concentration of free Mg^{2+} constant for ADP chases. The symbols for the final concentrations of added ADP are indicated to the right of the figure. The reaction was stopped with acid at the times indicated. The lines show the fit of a Pickart-Jencks model to the data with kinetic constants given in Table III, line 1. The data point in parentheses () was not used in fitting the model. The equation of the model is: $\mathrm{EP} = 100 \times (1 - \mathrm{extent}) \times e^{[-k(\mathrm{off}) \times \mathrm{extent} \times \mathrm{time}]}$ where $\mathrm{extent} = 1/(1 + \mathrm{K(internal)}\{1 + K_S(\mathrm{ADP})/[\mathrm{ADP}]\})$.

TABLE III

Action of Na^+ on Dephosphorylation of E1P by ADP

[Na^+]	Replacement cation	Pickart-Jencks constants; SEM			E1P	Enzyme prep.
		k(off)	K_S(ADP)	*K* (internal)		
mM		*/s*	*mM*		%	
500	none	1.1 ± 0.10	4.9 ± 2.2	0.10 ± 0.05	95	A
500	none	1.3 ± 0.20	1.9 ± 0.5	0.30 ± 0.09	95	A
200	Arg^+	1.9 ± 0.21	5.8 ± 0.5	1.4 ± 0.19	97	B
45	Arg^+	1.5 ± 0.36	4.4 ± 0.5	2.9 ± 0.77	97	A
200	NMG^+*	0.85 ± 0.24	1.6 ± 0.3	0.9 ± 0.11	79	C
45	NMG^+	0.69 ± 0.02	1.8§	2.1 ± 0.25	80	C
200	Et_3N^+	0.73 ± 0.07	1.2 ± 0.2	0.51 ± 0.06	56	B
45	Et_3N^+	0.78 ± 0.10	1.0 ± 0.1	1.3 ± 0.17	68	A

The experimental conditions were similar to those in Fig. 7. The sum of monovalent cation concentrations was 500 mM in all cases. Experimental procedure is in the legend of Fig. 7. Parameters are defined under Effect of Na^+ on Transphosphorylation from E1P to ADP. *NMG, *N*-methylglucamine. Et_3N, triethylamine. §The Jacobian was singular.

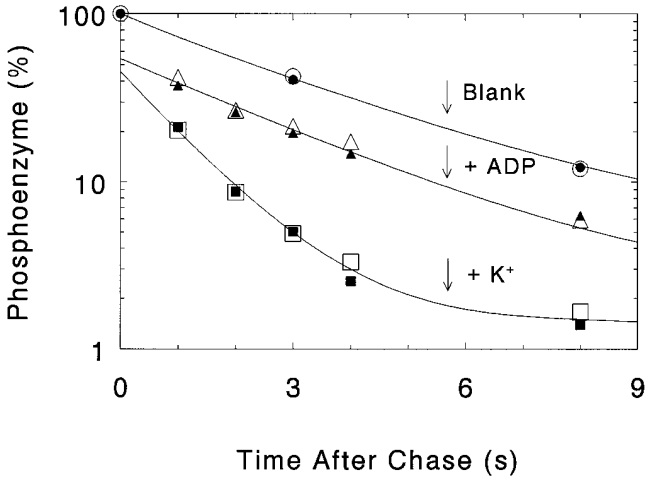

FIGURE 8. Test of additivity of Hofmeister effects by comparison of chloride with a mixture of nitrate and acetate. The reaction mixture contained 0.4 M NaCl (*closed symbols*) or 0.12 M $NaNO_3$ and 0.28 M sodium acetate (*open symbols*). Na,K-ATPase was phosphorylated from [γ-^{32}P]ATP for 40 s. Phosphorylation was terminated with the optimal chase solution without (*circles*) or with 1.4 mM (1.4 μmol) ADP (*triangles*) or 6 mM (6 μmol) K^+ (*squares*) with anions corresponding to those in the initial mixture. Control chases were with both ADP and K^+ and these values (<3% of initial phosphointermediate) were subtracted from corresponding values of ADP chases. The reaction was stopped with acid at the times indicated. The initial amount of phosphointermediate was the same in the two reaction mixtures.

To test additivity of Hofmeister effects we replaced chloride with a mixture of a cosmotropic anion, acetate, and a chaotropic anion, nitrate. By repeated trials we found that a mixture of 3 parts of nitrate and 7 parts of acetate was an exact replacement for chloride (Fig. 8). A mixture of 1 part of nitrate and 2 parts of acetate was a close but not an exact replacement (not shown). Additivity was remarkably precise. The correlation coefficient of the 17 paired values was 0.997. The probability of difference between the populations from which the paired values were taken is less than 5% by *t* test. Additivity of the actions of ions implies that Hofmeister effects are a single-valued parameter. That is, all salt solutions can be ranked on a single scale with respect to their Hofmeister effects. This scale is different from the scale of ionic strength. It could be called "Hofmeister strength."

Hofmeister Effects on ATPase Activity

To test for Hofmeister effects on Na,K-ATPase activity at 37°C, we compared chloride with acetate, nitrate, thiocyanate, and perchlorate at two concentrations, 40 and 400 mM. Anion composition had no effect at 40 mM. Increasing the salt concentration to 400 mM had no effect in acetate or chloride but, at 400 mM, chaotropic ions limited activity in proportion to their chaotropic potency (Fig. 9 *A*). We repeated the test on Na-ATPase activity in the absence of K^+ with similar effects of chaotropic anions at 400 mM (Fig. 9 *B*). In this case activity with acetate and chloride was strongly stimulated by increasing the salt concentration from 40 to 400 mM presumably due to hydrolysis of E2P by a high concentration of Na^+, showing a slight K-like effect (Glynn, 1985; Glynn 1988; Cornelius, 1991). (Note the difference in scale of activity with and without K^+.)

The rate constant of dephosphorylation in a blank chase without ADP or K^+ is directly proportional to Na-ATPase activity (Skou, 1991). We tested the effect of anions on this rate constant at 4°C. Changing the anions had no effect at 40 mM. Unexpectedly, increasing the concentration of $NaNO_3$ from 40 to 400 mM inhibited dephosphorylation (not shown) rather than stimulating it as in Fig. 9 *B*. The increase in inhibition was due to the lower temperature. When we compared Na-ATPase activity at 37°C with the rate constant of a blank chase at 4°C at various concentrations of $NaNO_3$, we saw that inhibition by nitrate was more effective at the lower temperature (Fig. 10). To confirm this interpretation we estimated both Na-ATPase activity and rate constant at 4, 17, and 27°C. The activation energy (ΔH) for both measurements was 1.3-fold larger in 400 than in 40 mM $NaNO_3$ (not shown). According to Collins and Washabaugh (1985) (their page 354) ". . . Hofmeister interactions are favored more as the temperature of aqueous solutions is lowered toward 0°C, and this temperature dependence is diagnostic for Hofmeister interactions." Thus anions affected the phosphointermediate and ATPase activity in ways characteristic of Hofmeister effects. It is outside the scope of this article to speculate on the physical chemistry of Hofmeister effects except

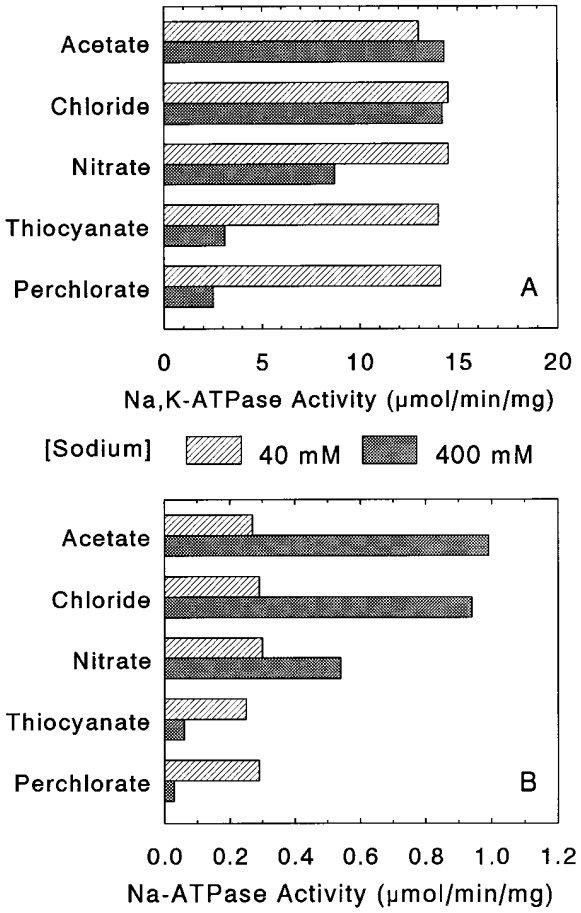

FIGURE 9. (*A*) Effect of anions on Na,K-ATPase activity at low and high salt concentrations. Activity of 0.045 U of Na,K-ATPase was estimated in 40 mM Na^+ and 8 mM K^+ with 3.5 mM Mg^{2+} (*light bars*) or in 400 mM Na^+ and 80 mM K^+ with 10 mM Mg^{2+} (*dark bars*). The anions were as indicated in the figure. Data with formate were like those with acetate (not shown). (*B*) Effect of anions on Na-ATPase activity in the absence of K^+ at low and high salt concentrations. Activity of 0.29 U of Na,K-ATPase was estimated in 40 mM Na^+ with 3.5 mM Mg^{2+} (*light bars*) or in 400 mM Na^+ with 10 mM Mg^{2+} (*dark bars*). The anions were as indicated. Formate was like acetate (not shown).

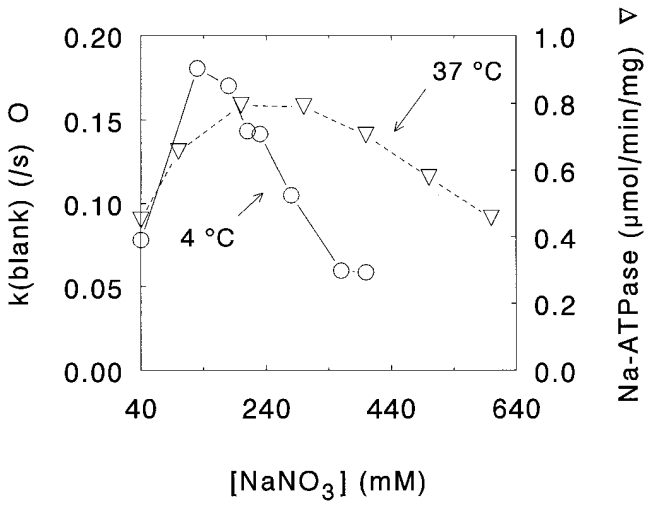

FIGURE 10. Comparison of rate constant of blank chase at 4°C with Na-ATPase activity at 37°C at various [$NaNO_3$]. At the same temperature these types of data can be superimposed by adjustment of scales. Increasing concentrations of $NaNO_3$ are more inhibitory at the lower temperature.

to comment that Hofmeister effects on protein folding are known (Collins and Washabaugh, 1985).

Fig. 7 in Post and Suzuki (1991) shows effects of a Hofmeister series of anions on rate constants of blank chases. In retrospect, these data should be normalized with reference to activity from 16 to 48 mM since there were no anion effects at 40 mM [salt] in Fig. 9 *B* and in the blank chases above. A redrawn figure is available from the corresponding author.

DISCUSSION

To investigate how Na^+ reacts with the phosphointermediate of Na,K-ATPase we studied equilibria. We assumed that when a change in concentration of Na^+ displaces an equilibrium between two species, then Na^+ binds preferentially to one of the species in the equilibrium.

We investigated two reactions. One was an equilibrium between the principal forms of the phosphorylated intermediate, E1P $\rightleftharpoons$ E2P. The other was an equilibrium of transphosphorylation, ATP·E1 $\rightleftharpoons$ ADP·E1P. An increase in [Na^+] had little effect on the first equilibrium but favored ATP·E1 over ADP·E1P in the second equilibrium. We concluded that Na^+ binds about equally strongly to E1P and E2P and binds more strongly to a complex of ATP with the dephosphoenzyme than to E1P.

E1P and E2P are thought to be different conformations of the phosphorylation domain of one molecule since the site of phosphorylation is the same aspartyl residue in both reactive states (Post et al., 1969; Siegel, et al., 1969; Post et al., 1975).

Definition of E1P

Some authors define the transition from E1P to E2P not in terms of reactivity of the phosphate group but in terms of release of occluded Na^+ to the medium in contact with the extracellular face of the membrane (Pedemonte, 1988; Fendler et al., 1993; Wuddel and Apell, 1995). It may be helpful to keep in mind the possibility of diverse definitions of a single expression. As defined by reactivity of the phosphate group, E1P and E2P are distinct since K^+ did not affect E1P and ADP did not affect E2P as shown by delayed chases (Figs. 1–3). The diagnostic reactions are: E1P + ADP $\rightarrow$ E1 + ATP and E2P + H_2O + 2 K^+ $\rightarrow$ E2(K_2) + P_i.

Equilibrium between E1P and E2P

To estimate this equilibrium we used a 2-pool model or a twin 2-pool model (see *Kinetic Constants* in MATERIALS AND METHODS and APPENDIX). The twin 2-pool model fitted the data well (Figs. 1–3). These models distinguished effects due to changes in equilibrium from effects due to hydrolysis. Na^+ increased the proportion of E1P in the phosphointermediate but did this only by stimulating hydrolysis of E2P; it did not affect the equilibrium of interconversion significantly (Figs. 4 and 5) and so bound equally strongly to E1P and to E2P.

How can Na^+ bind equally strongly to E1P and E2P? Pedemonte (1988) offers a model that explains the effect. His model reproduces patterns of data on Na^+ efflux, Na-ATPase activity in the absence of K^+, and ADP: ATP exchange. In his model there are separate forms of the phosphointermediate that bind 1–3 Na^+ or 1–2 K^+ from the extracellular medium. In his model Na^+ binds not only to the three Na^+ sites on one form but also to the two K^+ sites on the other form. It binds to different sites with different affinities. Na^+ binds with high affinity not only to one of the Na^+ sites but also to one of the K^+ sites. The overall effect is that there is little action of $[Na^+]$ on the partition between the forms as $[Na^+]$ increases from 50 to 500 mM. At 500 mM only about 15% of the phosphointermediate binds 3 Na^+ (Fig. 6 in Pedemonte, 1988). Thus if one form corresponds to Na_2E1P and the other to Na_2E2P, then changing the $[Na^+]$ has little effect on the equilibrium between the forms.

In these experiments at 4°C the rate constant of conversion of E1P to E2P was slow, $k12 < 0.1/s$ (Tables I and II). This value is 6,000-fold smaller than the value found in experiments on the enzyme from the electric eel at 24°C, 600/s (Froehlich and Fendler, 1991). Under the same conditions, rate constants of the eel enzyme were only threefold more rapid than those of the kidney enzyme (Forbush and Klodos, 1991). The effect of temperature on this step in the reaction sequence is extraordinary and worthy of further investigation.

Actions of Na^+ on Rate Constants

Na^+ stimulated hydrolysis of E2P (Figs. 4 and 5). This is a weak K-like action (Glynn, 1985; Glynn 1988; Cornelius, 1991). Na^+ also stimulated both forward and backward rates of interconversion (Fig. 5). In our experiments it also stimulated hydrolysis of E1P (Fig. 4 and experiments not shown). In experiments from the Biophysics Institute in Aarhus, hydrolysis of E1P was so slow that an effect of Na^+ could not be estimated (Fig. 5 and experiments in Klodos and Nørby, 1987, to which we fitted a 2-pool or twin 2-pool model.) We do not know a reason for the difference in rates of hydrolysis of E1P. Stimulation of hydrolysis of E1P cannot be a K-like effect since K^+ was present at a saturating concentration in those experiments. Thus in addition to its K-like effect Na^+ had a nonspecific stimulatory effect.

Equilibrium between ATP·E and ADP·E1P

The rate constant of dissociation of ATP, k_{off}, was found to be 0.7–1.9/s (Table III). This range is consistent with values of 1.5–3.5/s at 0°C in reference (Klodos and Nørby, 1987) obtained by a different method. The rate of dissociation of ADP was estimated in two experiments like that in Fig. 7 (not shown). The phosphointermediate was chased with 2 mM ADP for 1 s. At this time the [ADP] was diluted to 0.25 or 0.1 mM by adding a solution lacking ADP and containing all the other reactants at the same concentrations as in the reaction mixture. The rate of dephosphorylation decreased to the value at the dilute concentration within 0.5 s at 12°C. This result supports the assumption in the Pickart-Jencks model (Pickart and Jencks, 1982) that ADP equilibrates rapidly with the phosphointermediate.

Na^+ affected the equilibrium of transphosphorylation between ATP·E1 and ADP·E1P. When E1P was stabilized by a Hofmeister effect of a chaotropic anion, a high concentration of Na^+ shifted the equilibrium of transphosphorylation away from a complex of ADP with the phosphoenzyme, ADP·E1P, toward a complex of ATP with the dephosphoenzyme, ATP·E1, (Table III). Specifically, K(internal) = [ADP·E1P]/[ATP·E1]. K(internal) decreased from ~2 to ~0.2 as $[Na^+]$ increased from 45 to 500 mM. These data are consistent with the following reaction:

$$\mathrm{ATP \cdot E1 \cdot Na_3} \rightleftharpoons \mathrm{ADP \cdot E1P \cdot Na_2 + Na^+}. \qquad (7)$$

This reaction implies that one Na^+ can be released from E1P before E1P changes to E2P. Release of one Na^+ is consistent with the same ratio (10-fold) for the increase in $[Na^+]$ and the decrease in K(internal). In these experiments the dissociation constant for ADP, K_S(ADP), was higher with the substitute cation Arg^+ than with the other cations (Table III). Arg^+ probably competes with ADP for its active site. The amount of E1P was lower in *N*-ethylglucamine$^+$ or triethylamine$^+$ than in Arg^+ or Na^+, probably because the former cations are less chaotropic than the latter cations (Fig. 8 in reference Post and Suzuki, 1991).

Relationship of Findings to Reaction Sequence of Na,K-ATPase

The sodium pump comprises separate domains for phosphorylation and for transport (Karlish et al., 1990). The reaction sequence links reactions in the phosphorylation domain to those in the transport domain. This sequence is usually presented with an incomplete notation for forms of the transport domain (Pedemonte, 1988; Cornelius, 1991; Jørgensen, 1992;

Glynn, 1993; Horisberger, 1994). To remedy this deficiency we use § and ¶ to represent forms of the transport domain that bind Na^+ and K^+ respectively and subscripts "cyt" and "ext" to show conformations in which the transport sites have access to the cytoplasm and the extracellular medium respectively. The occluded conformation is intermediate between these conformations and is indicated by parentheses () as is customary. Thus the occluded sodium conformation is shown as §(Na_3). This form is linked to E1P in the reaction sequence giving E1P|§(Na_3), where the bar "|" links the phosphorylation and the transport domains. This form is an accepted intermediate in the reaction sequence (Cornelius, 1991; Jørgensen, 1992; Glynn, 1993; Robinson and Pratap, 1993; Horisberger, 1994). Release of Na^+ from E1P[2] in Eq. 7 can be incorporated in the reaction sequence as follows:

$$\mathrm{ATP \cdot E1|\S_{cyt} \cdot Na_3 \rightleftharpoons ADP \cdot E1P|\S\,(Na_3) \rightleftharpoons}$$
$$\mathrm{ADP \cdot E1P|\S\,(Na_2)\ + Na^+_{ext}}. \qquad (8)$$

Presumably ADP is released and Na_2 becomes deoccluded giving: E1P|$\S_{ext} \cdot Na_2$ + ADP. Failure of [Na^+] to influence the equilibrium between E1P and E2P can fit into the reaction sequence as follows:

$$\mathrm{E1P|\S_{ext} \cdot Na_2 \rightleftharpoons E2P|\P_{ext} \cdot Na_2}. \qquad (9)$$

Presumably the next step is replacement of Na_2 by 2 K^+ as follows:

$$\mathrm{E2P|\P_{ext} \cdot Na_2 + 2\ K^+_{ext} \rightleftharpoons E2P|\P_{ext} \cdot K_2 + 2\ Na^+_{ext}}, \qquad (10)$$

followed by dephosphorylation and occlusion of K^+ as follows:

$$\mathrm{E2P|\P_{ext} \cdot K_2 \rightleftharpoons E2|\P\,(K_2)\ + P_i}. \qquad (11)$$

Apparent Discrepancies

There might appear to be a discrepancy between Pedemonte's (1988) model and phosphorylation data at 500 mM NaCl. In Pedemonte's model only about 15% of the phosphointermediate binds 3 Na^+ at 500 mM. Yet Hara and Nakao (1981), for instance, found about 50% of the phosphointermediate to be sensitive to ADP at 500 mM NaCl. This apparent discrepancy can be resolved by recognition that Na^+ equilibrates very rapidly, $k > 700\ s^{-1}$ at 20°C (Wuddel and Apell, 1995). Data in dephosphorylation experiments are usually collected later than 1 s after addition of ADP at 0°C. Thus any E1P·Na_3 removed by reaction with ADP can be replaced by reequilibration of the less occupied forms until all E1P has reacted with ADP.

Another apparent discrepancy is in ADP:ATP exchange by erythrocyte membranes. At 0°C there was no effect of [Na^+] from 150 to 450 mM (Kaplan and Kenney, 1985). In these experiments [Cl^-] was kept constant as choline$^+$ replaced Na^+ and E1P was the predominant species of phosphointermediate. Perhaps under these conditions Na^+ is not deoccluded from E1P at a significant rate relative to the rate of turnover of the enzyme so that ADP:ATP exchange is not inhibited at the lower concentrations of Na^+.

Hofmeister Effects

In designing these experiments we used Hofmeister effects (Post and Suzuki, 1991) to obtain desired amounts of E1P and E2P at zero time. We needed comparable amounts of E1P and E2P to estimate interconversion rate constants precisely. We needed a large amount of E1P to estimate the effect of various concentrations of ADP precisely. Hofmeister effects were independent of high concentrations of Na^+, which are often used to increase the amount of E1P in this reaction system (Fig. 6). We confirmed the nature of these Hofmeister effects by showing additivity of the action of ions (Fig. 8) and enhancement at low temperature (Fig. 10), which are characteristics of Hofmeister effects (Collins and Washabaugh, 1985). Furthermore, we showed that these effects can be significant in the ATPase activity of this enzyme (Fig. 9). Discussion of the physical chemistry of Hofmeister effects is outside the scope of this paper.

Nonlinear Decay Kinetics of Phosphointermediates

Complex decay kinetics of phosphointermediates have been observed repeatedly and were reviewed by Froehlich and Fendler (1991). Up to that time the kinetics were interpreted by a 3-pool model of a homogeneous enzyme, a series model. Froehlich and Fendler (1991) proposed that "a series model of phosphorylation is inadequate" and that "the alternative . . . is a parallel pathway scheme" of a heterogeneous enzyme. The twin 2-pool model is a parallel pathway scheme; it fitted the data well in the work presented here. Post and Klodos (1996) discuss heterogeneity of this enzyme.

APPENDIX

The Twin 2-pool Model

The twin 2-pool model consists of two similar independent 2-pool submodels as shown in Fig. 11. One, on the left, is labeled "RAPID" since it has rapid rate constants. It is shown with an upright typeface. The other, on the right, is labeled "SLOW" since it has slow rate constants. It is shown with an italic typeface. Common

[2]The occluded sodium conformation is unstable in the native enzyme; it is stabilized by diverse modifications to the enzyme. These modifications also inhibit conversion of E1P to E2P. However, inhibition of conversion by a Hofmeister effect does not stabilize the occluded sodium conformation.

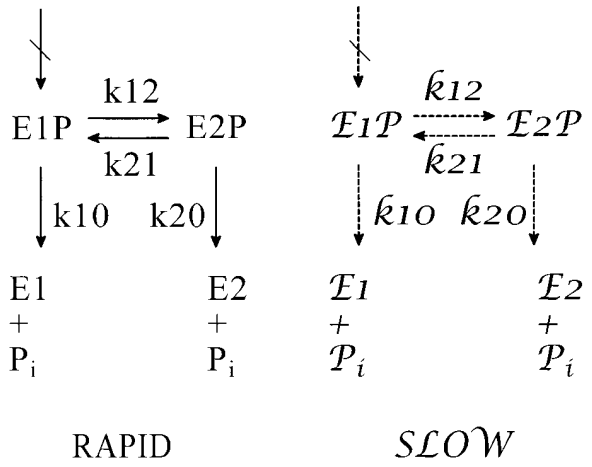

FIGURE 11. The twin 2-pool model. The model is described in the text.

characteristics of both submodels will be described first. Then the differences between the two submodels will be described.

Each submodel consists of two phosphointermediates, E1P and E2P. Binding of ligands to the intermediates is not a feature of the model. E1P and E2P are interconvertible. The rate constant for conversion of E1P to E2P is k12; the rate constant for conversion of E2P to E1P is k21. Each intermediate is subject to hydrolysis. The rate constant for hydrolysis of E1P is k10; The rate constant for hydrolysis of E2P is k20. The model applies to the behavior of the intermediates after their formation has been interrupted. During their formation before the interruption E1P is precursor to E2P. This feature is shown by the vertical arrow above E1P. The interruption of formation is shown by the slash (\) through the arrow. In both submodels E1P is resistant to K^+ and sensitive to ADP whereas E2P is resistant to ADP and sensitive to K^+.

The two submodels differ with respect to their rate constants. Each rate constant of the rapid submodel is larger than the corresponding rate constant of the slow submodel by a single factor. The difference in rates is shown by the difference in the arrows. The arrows of the rapid submodel are solid lines whereas the arrows of the slow submodel are dashed lines. Another difference between the submodels is the initial amount of phosphointermediate in each submodel after interruption of formation. The sum of E1P plus E2P in the rapid submodel can be different from the sum of E1P plus E2P in the slow submodel. This difference is not shown in the figure.

Fitting Data to the Twin 2-pool Model

Format of data for the experiment in Fig. 1. There are five (5) types of chase. A blank chase is started by addition of the optimal chase solution (1). A ligand chase is started by addition of K^+ (2) or ADP (3). In a standard ligand chase the ligand, K^+ or ADP, is added simultaneously with the optimal chase solution. In a delayed ligand chase the ligand is added after the optimal chase solution (4 and 5). Thus in a delayed chase the phosphointermediate undergoes two periods of decay. The first period is a blank chase. The second period is a ligand chase.

"x" and "y" are flags that tell the program whether the chase is a Blank, an ADP-chase, or a K^+-chase. "t1" is the duration of a blank chase. "t2" is the duration of a chase after addition of ADP or K^+. "ep" is the observed amount of phosphointermediate.

To save space only the first and last data points are shown for each type of chase (Table IV).

The fitting program. The variable parameters and their starting values are specified. The program has to start somewhere and the closer it starts to the final values the better. The starting values are simply guesses by the investigator.

Parameters: K10 = 0.075 K20 = 0.074 K12 = 0.078 K21 = 0.037 F = 0.59 h = 0.14 q = 6.0. K10, K20, K12, and K21 are the rate constants in Eq. 5.

F is the fraction of the initial amount of phosphointermediate at zero time that is the sum of the amounts of E1P in the rapid and slow submodels. h is the fraction of the initial amount of phosphointermediate at zero time that is in the slow submodel. (1 − h) is the fraction of the initial amount of phosphointermediate at zero time that is in the rapid submodel. q is the ratio of the values of the rate constants in the rapid submodel to the corresponding rate constants in the slow submodel.

Bounds. The limits over which the parameters can vary are specified.

TABLE IV

Format of Data Values to Fit Parameters of the Twin 2-pool Model to Data in the Experiment of Fig. 1

	Data Labels				
Chase Type	x	y	t1	t2	ep
Blank	0	0	2	0	87.50
Blank	0	0	40	0	10.22
ADP standard	1	0	0	2	34.34
ADP standard	1	0	0	44	3.35
ADP delayed	1	0	10	2	22.67
ADP delayed	1	0	10	28	4.80
K^+ standard	1	1	0	2	44.31
K^+ standard	1	1	0	44	2.63
K^+ delayed	1	1	10	2	18.50
K^+ delayed	1	1	10	28	4.19

Calibration factor qh. This factor specifies the relationship of the reported values of the rate constants to the values in the rapid and slow submodels. In this case the reported rate constants are those of a plain 2-pool model having the same ATPase activity as the combination of the rapid and slow submodels. The calibration is performed by modifying the values of the times. t1c and t2c are changed values of t1 and t2.

$$qh = [1 - h*(1 - 1/q)]; \; t1c = t1/qh; \; t2c = t2/qh.$$

The equations of the model. M0, M1, and M2 are intermediate values of rate constants. C1, C2, C3, and C4 are intermediate values of amounts of components of the phosphointermediate. * is a multiplication sign.

$$M0 = \text{square\_root}\{[(K10 + K12) - (K20 + K21)]^2 + (4*K21*K12)\}$$

$$M1 = (-K10 - K12 - K20 - K21 + M0)/2$$

$$M2 = (-K10 - K12 - K20 - K21 - M0)/2$$

$$C1 = [K21*(1 - F) - (K10 + K12 + M2)*F]/M0; \quad C2 = F - C1$$

$$C3 = [K12*F - (K20 + K21 + M2)*(1 - F)]/M0; \quad C4 = 1 - F - C3$$

E1R and E2R are the amounts of E1P and E2P in the rapid submodel, respectively. E1S and E2S are the amounts of E1P and E2P in the slow submodel, respectively. t1c determines the amounts at the end of the blank chases.

$$E1R = (1 - h)*\{[C1*\exp(tlc*M1)] + [C2*\exp(tlc*M2)]\}$$

$$E2R = (1 - h)*\{[C3*\exp(tlc*M1)] + [C4*\exp(tlc*M2)]\}$$

$$E1S = h*\{[C1*\exp(tlc*M1/q)] + [C2*\exp(tlc*M2/q)]\}$$

$$E2S = h*\{[C3*\exp(tlc*M1/q)] + [C4*\exp(tlc*M2/q)]\}$$

t2c determines the amounts at the end of the ligand chases.

$$E1R = E1R*\exp[-t2c*(K10 + K12)]; \quad E2R = E2R*\exp[-t2c*(K20 + K21)]$$

$$E1S = E1S*\exp[-t2c*(K10 + K12)/q]; \quad E2S = E2S*\exp[-t2c*(K20 + K21)/q]$$

$$E1 = E1R + E1S; \; E2 = E2R + E2S$$

$$ep = [(E1 + E2)*(1 - x)*(1 - y) + E2*x*(1 - y) + E1*x*y]*100$$

Action of the Program

The program uses the values of the variable parameters to calculate a fitted value of ep for each data point and the difference between the fitted value and the observed value. It combines these differences to find an error for all the data points taken together. Then it selects one of the parameters, changes it a little and calculates the error again. If the error is smaller, it repeats the process until no further improvement is found. If the error is larger, it makes a change in the opposite direction and repeats the process until no further improvement is found. It selects another parameter and repeats the process. In this way it optimizes the value of each parameter in turn repeatedly until the error can be made no smaller. If a true minimum error is obtained, the program reports that the result converged and is satisfactory. The program reports the values of the variable parameters that gave the best fit. If the program does not converge, the investigator has a problem that requires further study.

To plot fitted values of the phosphorylated intermediates we used values provided by the fitting program. To plot fitted values of E1P and E2P during the blank chase we wrote a program in BASIC to calculate these values using the parameters provided by the fitting program. A copy of this BASIC program is available from the corresponding author upon request.

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