Simulation of Dephosphorylation Curves by an Ensemble of Enzyme Molecules with Different Rate Constants

Dear Sir,

More than twenty years ago the Albers-Post scheme was proposed to describe the effects of ATP, ADP, Na, and K on transphosphorylation reactions carried out by Na,K-ATPase preparations. The scheme in its currently amplified version has been remarkably successful in accounting for the results of ion transport and transphosphorylation measurements, of physical and biochemical studies of the changes in pump configuration during transport cycles, of measurements of electrical events occurring during ion transport, and of measurements of the occlusion of Na and Rb ions by Na,K-ATPase preparations (Glynn, 1985, 1988). Basically, the Albers-Post scheme postulates that Na promotes phosphorylation of the pump by ATP to form the phosphorylated intermediate E₁P, that Na is released during the conformational transition from E₁P to E₂P, that K promotes the dephosphorylation of E₂P, and that K is released during the conformational transition from E₂ to E₁:

\[
\begin{align*}
ATP & \overset{\text{ADP}}{\rightarrow} E_1 \\
E_1 & \overset{\text{Na}}{\rightarrow} E_1P \\
E_1P & \overset{\text{Na}}{\rightarrow} E_2P \\
E_2P & \overset{\text{K}}{\rightarrow} E_2 \\
E_2 & \overset{\text{K}}{\rightarrow} E_1
\end{align*}
\]

Scheme 1

Nørby et al. (1983) reported the results of dephosphorylation experiments which raised several important questions. In these experiments, which were performed at 0°C, enzyme was phosphorylated by ATP at several concentrations of Na, and then dephosphorylation was initiated by the addition of K or ADP. Dephosphorylation was biphasic in the presence of either K or ADP. The slow phase of the dephosphorylation curves was extrapolated to the ordinate to estimate either the amount of K-sensitive intermediate (E₂P) present in the original equilibrium mixture from the dephosphorylation curves in the presence of K, or the amount of ADP-sensitive intermediate (E₁P) present in the original equilibrium mixture from the dephosphorylation curves in the presence of ADP. The sum of E₁P and E₂P at intermediate Na concentrations was greater than 100% of total EP, which suggested the existence of a phosphorylated intermediate between E₁P and E₂P. The presence of such a phosphorylated intermediate has been verified by other investigators (Yoda and Yoda, 1986) and by other methods (Lee and Fortes, 1985; Taniguchi et al., 1986), and its existence is now widely accepted.

Nørby et al. (1983) fit their dephosphorylation data to a reasonable model and calculated the maximum steady-state Na,K-ATPase activity which would be expected.
if the steps from $E_2P$ to $E_2P$ are rate limiting. The calculated activity was significantly
less than the activity measured experimentally under the conditions of the dephosphorylation experiments. These findings pose a serious challenge to the Albers-Post
model, and we wish to address that challenge in this communication.

At the Fifth International Conference on Na,K-ATPase in 1987, Forbush described
experiments similar to those of Nørby et al. (1983), but in Forbush's experiments
enzyme was phosphorylated at relatively high Na concentration, and dephosphorylation
was initiated by simultaneous addition of K and dilution of Na. The dephosphorylation
curves resembled curves from experiments in which both phosphorylation
and dephosphorylation were carried out at the lower rather than the higher Na
concentration. Subsequently, experiments were performed in which enzyme was
phosphorylated at relatively high Na, and dephosphorylation initiated by addition of
K. After the slow phase of dephosphorylation was reached, Na was diluted and there
was an immediate decrease in $EP$; the residual $EP$ then dephosphorylated at the rate
of the slow phase of enzyme phosphorylated at the lower Na concentration (Klodos
and Forbush, 1990). Apparently, reduction of Na increased the rate of transition of
$E_2P$ to $E_2P$. These findings did not seem to be explicable by the original model or, in
fact, by any conventional model.

![Figure 1](image-url)

**Figure 1.** Reaction scheme used for calculating dephosphorylation curves. $k_1$ and $k_5$ are
dependent on $[K^+]$ and $[ADP]$, respectively.

Kinetic models implicitly assume that the enzyme preparation is a homogeneous
population of molecules and the rate constant for each step is so narrowly distributed
about its mean that the distribution can be ignored. It occurred to us that it might be
possible to provide a qualitative explanation for the results of the dephosphorylation
experiments by assuming that the enzyme is not homogeneous, but is composed of
an ensemble of subgroups, that the rate constant for each step is narrowly distributed
in each of the subgroups, but that the mean rate constant for one or more of the
steps differs from subgroup to subgroup. Alternatively, we could assume that the rate
constant for one or more of the steps is broadly distributed about its mean, rather
than that the enzyme is made up of an ensemble of subgroups. That, however, would
greatly complicate our evaluation of the model, and our purpose is not to develop a
formal model with which to fit the data, but rather to propose a qualitative scheme
that accounts for some of the characteristics of the experimental data.

We examined the behavior of the simple scheme shown in Fig. 1, which is a
somewhat expanded version of a portion of the Albers-Post model. The model shows
two steps in which Na is released from the enzyme separated by a conformational
change (in which Na moves from position 1 to position 2). In the course of our
investigations we determined that an enzyme preparation composed of three or more
subgroups could yield results with this model that were consistent with experimental
observations. We arbitrarily decided that five subgroups should be sufficient to obtain
A good fit to the reported data. The subgroup rate constants and fractional compositions shown in Table I were chosen so as to closely reproduce the biphasic characteristics and rates of the dephosphorylation curves reported by Nørby et al. (1983).

We simulated the dephosphorylation experiments by first calculating for each subgroup the equilibrium distribution of the enzyme forms with both \( k_4 \) and \( k_5 \) set at zero. We then simulated K-induced dephosphorylation by rapidly changing \( k_4 \) from zero to the value shown in Table I, and ADP-induced dephosphorylation by rapidly changing \( k_5 \) from zero to the value shown. The results of the K-induced dephosphorylation are shown in the left-hand panel of Fig. 2, and the results of the ADP-induced dephosphorylation are shown in the right-hand panel. The results are similar to

| Enzyme subgroup | 1  | 2  | 3  | 4  | 5  |
|-----------------|----|----|----|----|----|
| Fraction of total | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 |
| \( k_1, \text{s}^{-1} \) | 25 | 25 | 25 | 25 | 25 |
| \( k_1, \text{s}^{-1} M^{-1} \) | 1,667 | 3,333 | 6,637 | 13,333 | 25,000 |
| \( k_2, \text{s}^{-1} \) | 40 | 40 | 40 | 40 | 40 |
| \( k_2, \text{s}^{-1} M^{-1} \) | 1 | 2 | 4 | 10 | 24 |
| \( k_3, \text{s}^{-1} \) | 50 | 50 | 50 | 50 | 6.7 |
| \( k_3, \text{s}^{-1} M^{-1} \) | 20 | 20 | 20 | 20 | 20 |
| \( k_4, \text{s}^{-1} \) | 10 | 10 | 10 | 10 | 10 |
| \( k_4, \text{s}^{-1} M^{-1} \) | 10 | 10 | 10 | 10 | 10 |

Figure 2. K\(^{+}\)-induced dephosphorylation (A) and ADP-induced dephosphorylation (B). In each case, the equilibrium distribution of phosphorylated species defined by Fig. 1 was determined using the rate constants and subgroup composition given in Table I and setting \([\text{ADP}] = [\text{K}] = 0\) (\( k_4 \) and \( k_5 \) set at zero). Dephosphorylation through step \( k_4 \) (K-induced, panel A) was simulated by setting \( k_4 \) at 10 \( \text{s}^{-1} \) at \( t = 0 \), and dephosphorylation through step \( k_5 \) (ADP-induced, panel B) was simulated by setting \( k_5 \) at 10 \( \text{s}^{-1} \) at \( t = 0 \).
those described by Nørby et al. (1983) in that the K- and ADP-induced dephosphorylation curves at each Na concentration show a rapid phase of dephosphorylation followed by a slow phase, and the extent of the rapid phase is dependent on the Na concentration. The different Na affinities at the two Na release steps and the separation of the release steps by a conformational change account in part for the biphasic dephosphorylation curves, but the marked biphasic behavior and the exact shape of the dephosphorylation curves arise from the assumption of enzyme heterogeneity. (Froehlich et al. [1983] have also pointed out that enzyme heterogeneity can result in biphasic dephosphorylation curves.) The model did not reproduce the findings that the sum of E₁P and E₂P calculated from the ordinate intercept of the

**Figure 3.** K⁺-induced dephosphorylation and Na⁺ dilution. The equilibrium distribution of phosphorylated species at [Na⁺] = 0.6 M was calculated as described in Fig. 2. In A, dephosphorylation was simulated by changing k₄ from 0 to 10 s⁻¹ at t = 0 and [Na⁺] was simultaneously reduced to 0.02 M. In B, dephosphorylation was simulated by changing k₄ from 0 to 10 s⁻¹ at t = 0, and [Na⁺] was reduced to 0.02 M 4 s later. In C, dephosphorylation was simulated by changing k₄ from 0 to 10 s⁻¹ at t = 0 and [Na⁺] was reduced to 0.15 M at t = 0 and to 0.02 M 4 s later.

The slow phase of the curves is greater than 100% of total EP, nor did we try to modify the model to reproduce that finding.

Fig. 3 shows the results of simulations in which we changed the Na concentration at the same time that K was added or at varying times after the addition of K. In panel A, Na was reduced at the same time that K was added; in panel B, Na was reduced 4 s after the addition of K; and in panel C Na was reduced at the same time that K was added, and then reduced again 4 s later. In all cases, reduction of Na resulted in rapid dephosphorylation to levels comparable to those seen in Fig. 2 when phosphorylation and dephosphorylation were carried out at the lower Na concentration. The slow phase of dephosphorylation was also similar to that seen when phosphorylation
and dephosphorylation were carried out at the lower Na concentration. Fig. 4 shows the results of a simulation in which enzyme was phosphorylated at 0.6 M Na, and then dephosphorylated by the simultaneous addition of K and reduction of Na to the final concentrations indicated at the right. These simulations result in dephosphorylation curves which appear to be similar to those described by Klodos and Forbush (1990).

It seemed to us that this model would qualitatively predict steady-state ATPase activity sufficient to account for the observed activity, while at the same time predicting the slow phase of dephosphorylation. The rapidly dephosphorylating subgroups at each Na concentration turn over rapidly during the steady-state measurements, while the slowly dephosphorylating subgroups account for the slow phase of dephosphorylation, but contribute little to the steady-state measurements. To test the prediction, we derived a steady-state equation for ATPase activity based on the sequence shown in Fig. 5. To make the sequence shown in Fig. 1 rate limiting for overall ATPase activity, we set \( k_4 \) and \( k_{-5} \) very large relative to the other rate constants. The circles in Fig. 5 represent ATPase activity calculated from the steady-state rate equation using the fractional contribution of enzyme subgroups and rate constants from Table I. The squares are ATPase activity calculated from Nørby et al. (1983), who measured activity under conditions similar to those used in the dephosphorylation experiments. At all Na concentrations, ATPase activity calculated from the model is more than adequate to account for the experimentally determined value. At each Na concentration, subgroups with the slowest rate of transition from \( E,PNa_3Na_5 \) to \( E,P \) account for the slow phase of dephosphorylation and the subgroups with the most rapid rate of transition account for the rapid phase of dephosphorylation and for most of the steady-state ATPase activity. Using this model, there is no reason to doubt that \( E,P \) is an intermediate in the overall ATPase reaction, and there is no reason to discard the Albers-Post model.

![Figure 4. K⁺-induced dephosphorylation and Na⁺ dilution. The equilibrium distribution of phosphorylated species at [Na⁺] = 0.6 M was calculated as described in Fig. 2. Dephosphorylation was simulated by changing \( k_5 \) from 0 to 10 s⁻¹ at \( t = 0 \) and [Na⁺] was simultaneously reduced to the values shown at the right.](image-url)
The model we use is complicated in that there are many rate constants. We do not believe that any simple model can simultaneously explain the dephosphorylation curves generated by addition of K, by addition of ADP, and by addition of K with dilution of Na either simultaneously or after a delay. No model in which phosphorylated intermediates interconvert in sequence along a single pathway seems likely to reproduce the dephosphorylation curves no matter how many rate constants are involved. The reaction mechanism may be even more complicated than our model; effects similar to those attributed to Na dilution can be produced experimentally by replacement of Cl with less lyotropic anions (Post, 1988; Klodos, 1990). If these

effects are due in part or solely to the dilution of Cl ions, then perhaps the reaction mechanism in Fig. 1 should also show Cl interacting with the enzyme, while the remainder of the analysis would be unchanged. We certainly do not wish to insist that our model is unique or even likely; we only wish to point out that a model with parallel pathways between E₁P and E₂P seems indispensable in accounting for the curves, and such models reconcile the dephosphorylation curves of Nørby et al. (1983) with the basic assumption of the Albers-Post scheme, that E₁P is an intermediate in the overall Na,K-ATPase mechanism.

Figure 5. Steady-state ATPase activity. The circles represent ATPase activity calculated from the reaction mechanism given in the inset and the subgroup distribution and rate constants given in Table I. To make the sequence from Fig. 1 rate limiting, \( k_4 \) and \( k_5 \) were set very large relative to the other rate constants. The squares are experimentally determined ATPase activity calculated from Nørby et al. (1983).
We suggest that some of the rate constants vary from molecule to molecule within a kinetic pool in an ATPase preparation. Heterogeneity of rate constants has been suggested for other proteins such as myoglobin at low temperatures and in solid samples (Austin et al., 1975), apoferritin (Rosato et al., 1987), phospholipase A2 (Ludescher et al., 1985), and ribonuclease T (Gryczynski et al., 1988). Moreover, many environmental manipulations have been found to alter the distribution of phosphorylated intermediate between E1P and E2P: temperature (Kaplan and Kenney, 1985), cholesterol and the ionophores monensin, nigericin, and A23187 (Yoda and Yoda, 1987), lyotropic anions (Post, 1988), etc. Since these environmental manipulations alter the EIP/E2P ratio, they must alter at least one of the rate constants between E1P and E2P. Since ATPase preparations used in dephosphorylation experiments consist of enzyme embedded in a phospholipid membrane, it is not unreasonable to suppose that variations in the local environment of each molecule may modify its kinetic characteristics. Kinetic heterogeneity in a relatively pure ATPase preparation has been discussed by Forbush (1987) and directly demonstrated by Suzuki and Post (1990). Variations in the local phospholipid environment of ATPase molecules, particularly at low temperature, has been proposed as a cause of significant kinetic heterogeneity (Klodos and Forbush, 1990; Suzuki and Post, 1990).

We suggest that the possibility of kinetic heterogeneity and parallel reaction pathways be considered when membrane-embedded molecules appear to behave anomalously.

This work was supported by United States Public Health Service grant DK-19185.

Original version received 17 October 1990 and accepted version received 22 March 1991.

REFERENCES

Austin, R. H., K. W. Belson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. Biochemistry. 14:5355-5373.

Forbush, B., III. 1987. Rapid release of 42K and 86Rb from an occluded state of the Na,K-pump in the presence of ATP or ADP. Journal of Biological Chemistry. 262:11109-11115.

Froehlich, J. P., A. S. Hobbs, and R. W. Albers. 1983. Evidence for parallel pathways of phosphoenzyme formation in the mechanism of ATP hydrolysis by Electrophorus Na,K-ATPase. Current Topics in Membranes and Transport. 19:513-535.

Glynn, I. M. 1985. The Na+,K+-transporting adenosine triphosphatase. In The Enzymes of Biological Membranes. Vol. 3. 2nd ed. A. N. Martonosi, editor. Plenum Publishing Corp., New York. 35-114.

Glynn, I. M. 1988. The coupling of enzymatic steps to the translocation of sodium and potassium. In The Na+,K+-Pump, Part A: Molecular Aspects. J. C. Skou, J. G. Nørby, A. B. Maunsbach, and M. Esmann, editors. Alan R. Liss, Inc., New York. 435-460.

Gryczynski, I., M. Eftink, and J. R. Lakowicz. 1988. Conformation heterogeneity in proteins as an origin of heterogeneous fluorescence decays, illustrated by native and denatured ribonuclease T. Biochimica et Biophysica Acta. 954:244-252.

Kaplan, J. H., and L. J. Kenney. 1985. Temperature effects on sodium pump phosphoenzyme distribution in human red blood cells. Journal of General Physiology. 85:123-136.

Klodos, I. 1990. Effect of lyotropic anions on the dephosphorylation of Na,K-ATPase phosphointermediates. Journal of General Physiology. 96:53a-54a. (Abstr.)

Klodos, I., and B. Forbush III. 1990. Transient kinetics of dephosphorylation of Na,K-ATPase after dilution of NaCl. Journal of General Physiology. 96:52a-53a. (Abstr.)
Lee, J. A., and P. A. G. Fortes. 1985. Anthroyl-ouabain binding to different phosphoenzyme forms of Na,K-ATPase. In The Sodium Pump. I. Glynn and C. Ellory, editors. The Company of Biologists Ltd., Cambridge, UK. 277–282.

Ludescher, R. D., J. J. Bolwerk, G. H. de Haas, and B. S. Hudson. 1985. Complex photophysics of the single tryptophan of porcine pancreatic phospholipase Aα, its zymogen, and its enzyme/micelle complex. Biochemistry. 24:7240–7249.

Nørby, J. G., I. Klodos, and N. O. Christiansen. 1983. Kinetics of Na-ATPase activity by the Na,K pump. Interaction of the phosphorylated intermediates with Na⁺, Tris⁺ and K⁺. Journal of General Physiology. 82:725–759.

Post, R. L. 1988. Lyotropic ions on reactivity of phosphoenzyme of sodium, potassium-ATPase. Biophysical Journal. 53:138a. (Abstr.)

Rosato, N., A. Finazzi-Agro', E. Gratton, S. Stefanini, and E. Chiancone. 1987. Time-resolved fluorescence of apoferritin and its subunits. Journal of Biological Chemistry. 262:14487–14491.

Suzuki, K., and R. L. Post. 1990. Heterogeneous kinetics of the phosphoenzyme of Na,K-ATPase. Journal of General Physiology. 96:57a. (Abstr.)

Taniguchi, K., K. Suzuki, T. Sasaki, H. Shinya, and S. Iida. 1986. The change in light scattering following formation of ADP-sensitive phosphoenzyme in Na⁺, K⁺-ATPase modified with N-[p-(2-benzimidazolyl)phenyl] maleimide. Journal of Biological Chemistry. 261:3272–3281.

Yoda, S., and A. Yoda. 1986. ADP- and K⁺-sensitive phosphorylated intermediate of Na,K-ATPase. Journal of Biological Chemistry. 261:1147–1152.

Yoda, S., and A. Yoda. 1987. Phosphorylated intermediates of Na,K-ATPase proteoliposomes controlled by bilayer cholesterol. Interaction with cardiac steroid. Journal of Biological Chemistry. 262:103–109.

Dwight W. Martin
John R. Sachs
Department of Medicine,
State University of New York at Stony Brook,
Stony Brook, New York 11794-8151