Phosphorylation of the Sodium−Potassium Adenosinetriphosphatase with Adenosine Triphosphate and Sodium Ion That Requires Subconformations in Addition to Principal E_1 and E_2 Conformations of the Enzyme†

Manik C. Ghosh‡ and William P. Jencks*

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110

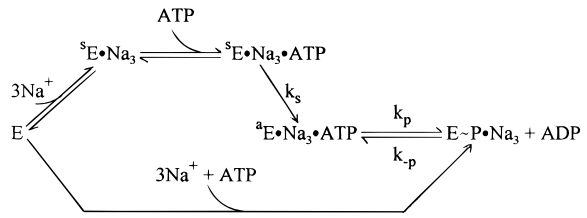
Received June 4, 1996⊗

ABSTRACT: Phosphorylation of the free sodium−potassium adenosinetriphosphatase of sheep kidney upon the addition of 0.02−2.0 mM ATP and a saturating concentration of Na^+ (125 mM) follows pseudo-first-order kinetics. The first-order rate constant increases with increasing [ATP] and levels off at high [ATP] with a limiting rate constant of 180 s^{-1} at 25 °C, pH 7.4, 125 mM NaCl, and 3.0 mM $MgCl_2$. This rate constant is about $^1/_3$ of the maximum rate constant of 460 s^{-1} for phosphorylation of enzyme that had been preincubated with Na^+ under identical conditions [Keillor, J. W., & Jencks, W. P., (1996) *Biochemistry 35*, 2750−2753]; this rate ratio is similar to that for phosphorylation of the calcium ATPase with and without initial incubation with Ca^{2+} [Petithory, J. R., & Jencks, W. P. (1986) *Biochemistry 25*, 4493−4497]. The $K_{0.5}$ for ATP is 18 ± 3 μM for the free enzyme, which is about $^1/_4$ of $K_{0.5}$ = 75 μM for enzyme that was preincubated with Na^+. Addition of ADP to ATP and Na^+ decreases the yield of E∼P progressively with increasing ADP concentration. Upon an increase of [ADP] from 0 to 2.0 mM, the rate constant for phosphorylation decreases 4-fold (167 to 41 s^{-1}) at low [ATP] (0.25 mM) and about 2.7-fold (174 to 64 s^{-1}) at high [ATP] (2.0 mM). The absence of an induction period for phosphorylation of E upon the addition of saturating concentrations of ATP and Na^+ indicates that all the prior reaction steps are much faster than the rate-limiting step. These results are consistent with a rate-determining conformational change of the E·ATP·Na_3 intermediate. The decrease of the rate constant with increasing [ADP] is attributed to competition between ATP and ADP for the free enzyme.

The sodium−potassium ATPase, also known as the sodium pump or sodium−potassium pump, is a membrane-bound enzyme that is responsible for maintaining the high internal potassium and low internal sodium ion concentrations that are characteristic of most animal cells. This P-type ATPase couples the hydrolysis of each molecule of ATP to the transport of three sodium ions out of the cell and two potassium ions into the cell. The electrochemical gradient that is produced in this process maintains the resting potentials of the cells, as well as the activity of muscle and nerve tissue, and provides the driving force for the translocation of glucose, amino acids, and other nutrients into cells. A number of reviews that describe properties of the sodium pump are available (Cantley, 1981; Kaplan, 1983; Glynn, 1985; Post, 1989; Glynn & Karlish, 1990; Skou & Esmann, 1992; Robinson & Pratap, 1993; Lingrel, 1994).

The reaction of Na^+,K^+-ATPase with its substrates ATP and Na^+ can occur by at least two pathways, depending on the order of mixing (Scheme 1). In the upper pathway, which has recently been studied by Keillor and Jencks (1996), the enzyme is preincubated with sodium. The Na^+-bound enzyme, designated as ^{s}E·Na_3 simply to indicate that it is the stable form of the enzyme with bound sodium, then binds ATP to give ^{s}E·Na_3·ATP as shown in the upper part of Scheme 1. The ^{s}E·Na_3·ATP undergoes a rate-limiting

Scheme 1



conformational change with a rate constant of k_s = 460 s^{-1} that converts the enzyme to the catalytically active form, ^{a}E·Na_3·ATP, which is phosphorylated very rapidly with an estimated rate constant of $k_p \geq 3000$ s^{-1}.

The mechanism of the lower pathway in Scheme 1, which is followed when ATP and Na^+ are added together to the enzyme, has not been fully characterized. We describe here the properties of this pathway, which are significantly different from those of the upper pathway in Scheme 1. An expected outcome of this study is an answer to the question, "Are the two principal conformations E_1 and E_2 (Albers, 1967; Post *et al.*, 1969, 1972) for the sodium−potassium ATPase adequate to explain our results and the coupling between ATP hydrolysis and the transport of Na^+ and K^+ ions, or does this coupling require additional subconformations of the enzyme?" (Norby, 1988; Glynn & Karlish, 1990; Pratap & Robinson, 1993).

MATERIALS AND METHODS

Materials. Reagents were generally of the highest purity available and were used without further purification. Sodium

† Publication No. 1806. This research was supported by Grant GM-20888 from the National Institutes of Health.

‡ Present address: Laboratory of Biochemistry, National Cancer Institute, Building 37, Room 4C-06, National Institutes of Health, Bethesda, MD 20892

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(96)01324-4 CCC: $12.00

salts of adenosine 5′-triphosphate and adenosine 5'-diphosphate were obtained from Boehringer Mannheim, and [γ-^{32}P]-ATP (specific activity = 30 Ci/mmol) was purchased from New England Nuclear.

Sodium-potassium adenosinetriphosphatase was isolated from sheep kidneys by a slight modification of the step gradient method of Jorgensen (1974). Sheep kidneys were collected within 0.5 h of exsanguination and were frozen immediately on dry ice. After dissection, the enzyme was purified following Jorgensen's method by treating with $^1/_4$ (w/w) of the equivalent amount of sodium dodecyl sulfate. These preparations catalyzed the hydrolysis of ATP with a rate of 3−5 μmol (mg of total protein)$^{-1}$ min^{-1}, and 1.0 mM ouabain inhibited 98−99% of the ATPase activity. We did not purify the enzyme extensively because membrane-bound enzymes may undergo changes in their catalytic properties in a non-physiological environment.

Methods. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin (BSA) as the protein standard. It may be noted that the Lowry method for estimation of the protein concentration of purified sodium−potassium ATPase gives slightly lower values than that obtained from amino acid analysis (Peters *et al.*, 1982). Sodium−potassium adenosinetriphosphatase activity was assayed spectrophotometrically by coupling ADP production to NADH oxidation using pyruvate kinase (PK) and lactate dehydrogenase (LDH) at 37 °C as described by Rossi *et al*. (1979). Standard conditions for the enzyme assay were pH 7.4 with 30 mM Tris buffer, 2.0 mM ATP, 1.5 mM phosphoenolpyruvate (PEP), 0.15 mM NADH, 0.05 mg/mL PK, 0.05 mg/mL LDH, 1.0 mM EDTA, 110 mM NaCl, 15 mM KCl, and 3.0 mM $MgCl_2$.

The formation of phosphoenzyme at 25 °C was followed by the mix−quench technique (Froehlich , 1976a,b; Stahl & Jencks, 1984) using a three-syringe KinTek rapid-mixing apparatus (Johnson, 1986). Temperature-equilibriated 15 μL samples of enzyme and of ATP plus Na^+ at 25 °C are driven from the sample loops by the buffer solutions in syringes A and B into a mixing chamber connected to a length of narrow-bore Teflon tubing and allowed to react for t = 2−25 ms. The reactions were quenched in a second mixing chamber with 1.5 N hydrochloric acid from syringe C. The reaction times were calibrated by measuring the known rate constants for hydrolysis of benzylidene malononitrile with hydroxide ion at 20 °C, and the tube lengths were calibrated with solutions of [γ-^{32}P]ATP.

Phosphorylation of the Na^+,K^+-ATPase was measured in a solution containing 3.0−10.0 mM $MgCl_2$ and 30 mM Tris buffer at pH 7.4. The enzyme was found to remain stable in this medium for more than 1 h at room temperature. A 15 μL aliquot of the enzyme was loaded into sample loop A, and 15 μL of [γ-^{32}P]ATP plus NaCl (with or without ADP) in the same buffer was loaded into sample loop B prior to each run. The reaction was initiated within 10 s of loading. The quenched reaction mixture was then mixed with 1 mg of BSA (acting as a carrier) and then precipitated with trichloroacetic acid (TCA) to give a final concentration of 12% TCA. The amounts of [^{32}P]E∼P in the quenched reaction mixtures were determined by the method of Verjovski-Almeida *et al*. (1978). E_{tot} was taken as the amount of phosphoenzyme formed after a long period (2 s) of reaction. Observed rate constants (k_{obs}) and the error limits were obtained by exponential fitting of the data (E∼P/E_{tot}) against time. The order of addition of substrate and quench was reversed to obtain blanks.

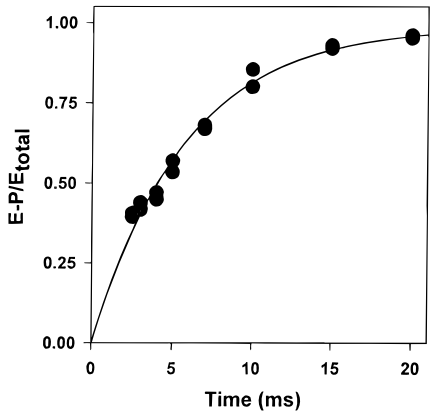


FIGURE 1: Formation of E∼P·Na_3 by reaction of E with 1.0 mM [γ-^{32}P]ATP and 125 mM Na^+. Final conditions were 3.0 mM $MgCl_2$, pH 7.4 (30 mM Tris), and 0.65 mg/mL sheep-kidney Na^+,K^+-ATPase at 25 °C. Syringe A contained 1.3 mg/mL ATPase, syringe B 2.0 mM [γ-^{32}P]ATP and 250 mM NaCl, and syringe C 1.5 M HCl. All other components were present in syringes A and B at the final concentrations. The best fit to a single exponential has a rate constant of 174 s^{-1}.

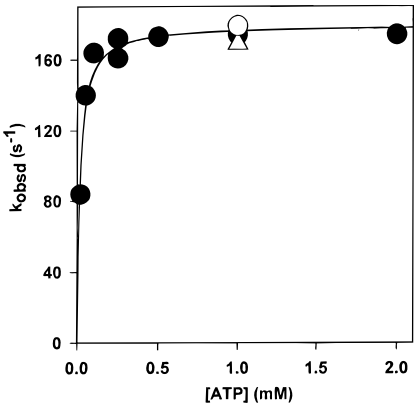


FIGURE 2: Dependence on ATP concentration of the first-order rate constants for phosphorylation of 0.65 mg/mL ATPase with 125 mM (filled circles) and 250 mM (open circle) NaCl, 0.02−2.0 mM [γ-^{32}P]ATP at 25 °C and pH 7.4 (30 mM Tris), and 3.0 mM (filled circles) and 10.0 mM (open triangle) $MgCl_2$. The line is drawn for K_m = 18 μM. The contents of the syringes were as described in Figure 1.

RESULTS AND DISCUSSION

Reaction of E with ATP and Na^+. Figure 1 shows that [^{32}P]E∼P·Na_3 is formed with an observed first-order rate constant of 174 s^{-1} when the sheep-kidney Na^+,K^+-ATPase, preincubated with 3.0 mM Mg^{2+}, is added to 1.0 mM [γ-^{32}P]-ATP and 125 mM NaCl at 25 °C and pH 7.4. The rate constant increases hyperbolically with increasing ATP concentration, and Figure 2 shows the fit of the data (Table 1, supporting information) to the Michaelis−Menten equation with k_{cat} = 180 s^{-1}, and a small $K_{0.5}$ of 18 μM. The values of E∼P/E_{tot} at different ATP concentrations fall in the range of 0.75−1.0 (Table 1 of supporting information). The negative deviation from 1.0 of E∼P/E_{tot} is not systematic with increasing ATP concentration and is probably the result of slow hydrolysis of E∼P. According to the E_1−E_2 nomenclature, E in the present system corresponds to the E_2 form of the enzyme, whereas E·ATP·Na_3 represents the E_1 form.

The absence of a lag phase in the phosphorylation of E in the presence of saturating concentrations of ATP and sodium ion indicates that only one step is rate-limiting. The rate-determining step does not represent phosphoryl transfer, k_p in eq 1,

$$E + (ATP + Na^+) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ATP{\cdot}Na_3 \xrightarrow{k_e} {}^aE{\cdot}ATP{\cdot}Na_3 \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} E{\sim}P{\cdot}Na_3 + ADP \quad (1)$$

because k_p has been indirectly estimated to be ≥ 3000 s^{-1} when ATP is added to $E{\cdot}Na_3$ under identical conditions (Keillor & Jencks, 1996). The results are consistent with a rate-limiting conformational change, with $k_e = 180$ s^{-1} (eq 1), which gives an activated species $^aE{\cdot}ATP{\cdot}Na_3$ that undergoes very rapid phosphorylation.

Essentially identical rate constants were obtained for the phosphorylation of E with a saturating concentration of 1.0 mM ATP and higher concentations of 250 mM Na^+ and 10 mM Mg^{2+} (open circle and triangle in Figure 2), in agreement with the earlier results of Mardh and Post (1977) for phosphorylation of the free enzyme under similar conditions. These authors also showed that addition of Mg^{2+} to the enzyme beforehand, separately or in combination with Na^+ or ATP, had little effect on the initial phosphorylation rate of the enzyme. A rate constant of $k_{max} = 460$ s^{-1} for the phosphorylation of Na^+-preincubated enzyme, $E{\cdot}Na_3$, with ATP has recently been reported from this laboratory (Keillor & Jencks, 1996); this rate constant is about 2.6 times larger than $k_{cat} = 180$ s^{-1} for phosphorylation upon addition of ATP and Na^+ to the free enzyme, in agreement with the earlier results of Mardh and Post (1977). This ratio is similar to the corresponding ratio found by Stahl and Jencks (1984, 1987) for the calcium pump, another member of the P-type ATPases.

Reaction of E with ATP and Na^+ in the Presence of Added ADP. The effect of added ADP on the reaction rate and the final yield of phosphoenzyme was examined in an effort to identify the rate-limiting step for the phosphorylation of E upon addition of ATP and Na^+ to the enzyme. If phosphoryl transfer were the rate-determining step, the observed rate constant for approach to the equilibrium concentration of phosphoenzyme in the presence of ADP would be equal to the sum of the first-order rate constants for phosphorylation and for phosphoryl transfer to ADP in the reverse direction, as shown in eq 2; i.e., $k_{obs} = k_f + k_r$ (Frost & Pearson, 1953).

$$E + (ATP + Na^+) \rightleftharpoons E{\cdot}ATP{\cdot}Na_3 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \begin{cases} E{\sim}P{\cdot}Na_3{\cdot}ADP \\ \Downarrow\Uparrow \\ E{\sim}P{\cdot}Na_3 + ADP \end{cases} \quad (2)$$

The magnitude of k_{obs} is then expected to increase with increasing concentration of added ADP. However, k_{obs} will not increase if some step other than phosphoryl transfer, i.e., a conformational change, is rate-determining. In the present system, with an increase in [ADP] from 0 to 2.0 mM, in the presence of 0.25 mM ATP, the maximum amount of phosphoenzyme formed decreases 3.5-fold, from 1.0 to 0.29, and k_{obs} decreases 4-fold, from 167 to 41 s^{-1}, as shown in Figure 3. The first-order rate constants for the phosphorylation of Na^+,K^+-ATPase preincubated with Na^+ (Keillor & Jencks, 1996) and of both free and Ca^{2+}-incubated

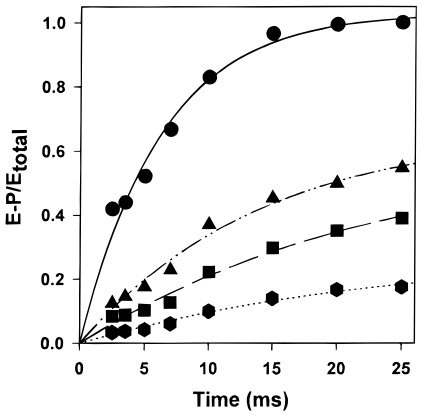


FIGURE 3: Phosphorylation of E with ATP and Na^+ in the absence and presence of added ADP. The final conditions were 0.65 mg/mL ATPase, 0.25 mM [γ-^{32}P]ATP, 125 mM NaCl, 3.0 mM $MgCl_2$, pH 7.4 (30 mM Tris), and 0 mM (filled circles), 0.5 mM (filled triangles), 1.0 mM (filled squares), and 2.0 mM (filled hexagons) ADP at 25 °C. Reactions were performed essentially as described in Figure 1, except that syringe B contained ADP in addition to [γ-^{32}P]ATP and Na^+. The calculated lines were drawn with rate constants of 167 (—), 71 (— - - —), 43 (- - -), and 41 (•••) s^{-1}.

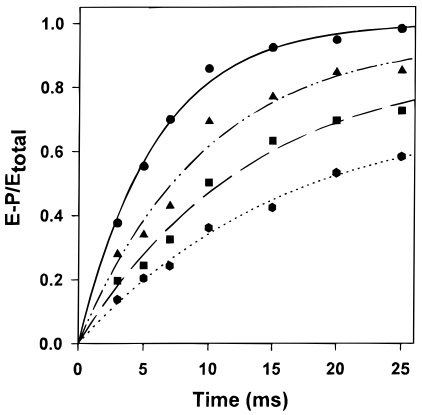
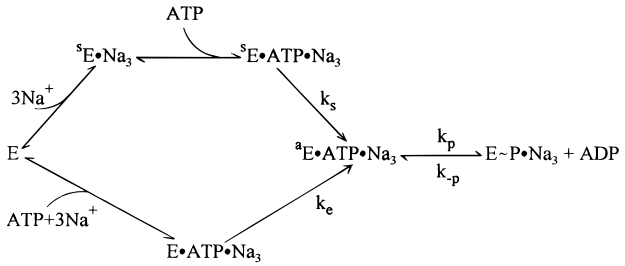


FIGURE 4: Phosphorylation of 0.65 mg/mL E with 2.0 mM [γ-^{32}P]-ATP and 125 mM NaCl in the absence (filled circles) and presence of 0.5 mM (filled triangles), 1.0 mM (filled squares), and 2.0 mM (filled hexagons) ADP. The other conditions and the contents of the syringes were as described in Figure 3. The calculated lines were drawn with rate constants of 174 (—), 104 (— - - —), 76 (- - -) and 63 (•••) s^{-1}.

Ca-ATPase (Petithory & Jencks, 1986; Stahl & Jencks, 1987) were found to remain unaffected by added ADP at a high saturating concentration of 1.0 mM ATP. In all three cases, it was concluded that the rate-limiting step is a conformational change to give an activated enzyme−substrate complex that undergoes very fast phosphoryl transfer. In the present system the decrease in the rate constant for phosphorylation of E with ATP and sodium ion in the presence of added ADP can be explained simply by competition between ATP and ADP for the free enzyme. This conclusion is supported by the results in Figure 4, which show that the extent of the decrease in the rate constant for phosphorylation is smaller at a higher concentration of ATP (2.0 mM). Due to the possibility of competitive inhibition by ADP, the [ADP] variation results do not conclusively eliminate the possibility of either of these two steps, conformational change and phosphoryl transfer, being rate-limiting. However, if phosphoryl transfer were rate-limiting, ADP should have two opposing effects on the observed first-order rate constant for phosphorylation: (1) a hyperbolic increase as a result of

Scheme 2



lowering of the infinity value and (2) a decrease as a result of competitive inhibition by ADP. The resulting rate constant would be a complicated function of [ADP].

Comparison of our results for phosphorylation of the free enzyme (E) upon addition of Na^+, ATP, and ADP with those for the reaction of Na^+-incubated enzyme with ATP and ADP (Keillor & Jencks, 1996) leads to the conclusion that ADP binds more strongly to the free enzyme than to the sodium-bound enzyme ($E\cdot Na_3$).

For the reactions of both free enzyme and Na^+-incubated enzyme, $E\sim P/E_{tot}$ increases with increasing [ATP] at a given concentration of ADP. This may be attributed to two opposing factors. (i) With an increase in [ATP], the free [ADP] increases because ATP chelates some of the free Mg^{2+} and converts MgADP to free ADP, which is a product of phosphorylation. This factor should cause a decrease in $E\sim P/E_{tot}$. (ii) With an increase in [ATP], both of the equilibria in eq 1 are shifted in the forward direction, which should result in an increase in $E\sim P/E_{tot}$. Our results indicate that factor (ii) dominates over factor (i).

All of these results are consistent with a rate-limiting conformational change of the $E\cdot ATP\cdot Na_3$ intermediate. Indirect evidence for a conformational change of the $E\cdot ATP\cdot Na_3$ complex prior to phosphoryl transfer for the reaction of E with ATP and Na^+ has been reported by Taniguchi *et al.* (1984, 1986, 1991). Fluorescence changes upon formation of E∼P from the free enzyme were interpreted by these authors in terms of a pathway that proceeds through an activated enzyme−substrate complex that is analogous to our $^{a}E\cdot ATP\cdot Na_3$.

The results reported here may be described by the model of Scheme 2. The principal difference between Scheme 2 and most models for the sodium−potassium ATPase is that in Scheme 2 there are two pathways for the reaction with ATP: the well-known pathway that involves initial binding of Na^+ with E to form a stable, high-affinity species $^{s}E\cdot Na_3$, followed by binding of ATP and phosphorylation (upper pathway, Scheme 2), and another pathway that involves fast binding of ATP and Na^+ before phosphorylation, followed by slower phosphorylation (lower pathway, Scheme 2). In both pathways, the rate-limiting step for phosphorylation upon addition of ATP is a conformational change to form the catalytically active species of the enzyme, $^{a}E\cdot ATP\cdot Na_3$, with a rate constant of $k_s = 460\ s^{-1}$ for reaction of $^{s}E\cdot ATP\cdot Na_3$ (top) or $k_e = 180\ s^{-1}$ for reaction of $E\cdot ATP\cdot Na_3$ (bottom), which is followed by very rapid phosphoryl transfer.

We conclude that subconformational states of the sodium−potassium ATPase, in addition to its two principal conformations E_1 and E_2, are required to explain these results.

ACKNOWLEDGMENT

The authors are grateful to Drs. Jeff Keillor and Jayhyuk Myung for stimulating discussions and thank E. L. Blood and Son of West Groton, MA, and their employees for their help in obtaining fresh sheep kidneys.

REFERENCES

Cantley, L. C. (1981) *Curr. Top. Bioenerg. 11*, 201−237.
Froehlich, J. P., Albers, R. W., Koval, G. J., Goebel, R., & Berman, M. (1976) *J. Biol. Chem. 251*, 2186−2188.
Froehlich, J. P., Sullivan, J. V., & Berger, R. L. (1976b) *Anal. Biochem. 73*, 331−341.
Frost, A. A., & Pearson, R. G. (1953) *Kinetics and Mechanism*, pp 154−173, Wiley, New York.
Glynn, I. M. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. M., Ed.) pp 35−114, Plenum Press, New York.
Glynn, I. M., & Karlish, S. J. D. (1990) *Annu. Rev. Biochem. 59*, 171−205.
Johnson, K. A. (1986) *Methods Enzymol. 134*, 677−705.
Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36−67.
Kaplan, J. H. (1983) *Am. J. Physiol. 245*, G327−G333.
Keillor, J. W., & Jencks, W. P. (1996) *Biochemistry 35*, 2750−2753.
Lingrel, J. B., & Kuntzweiler, T. (1994) *J. Biol. Chem. 269*, 19659−19662.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Mardh, S., & Post, R. L. (1977) *J. Biol. Chem. 252*, 633−638.
Peters, W. H., Fleuren-Jakobs, A. M., Kamps, K. M., DePont, J. J., & Bonting, S. L. (1982) *Anal. Biochem. 124*, 349−352.
Petithory, J. R., & Jencks, W. P. (1986) *Biochemistry 25*, 4493−4497.
Post, R. L. (1989) *Annu. Rev. Physiol. 51*, 1−15.
Robinson, J. D., & Pratap, P. R. (1993) *Biochim. Biophys. Acta 1154*, 83−104.
Rossi, B., Leone, F. de A., Gache, C., & Lazdunski, M. (1979) *J. Biol. Chem. 254*, 2302−2307.
Skou, J. C., & Esmann, M. (1992) *J. Bioenerg. Biomembr. 24*, 249−261.
Stahl, N., & Jencks, W. P. (1984) *Biochemistry 23*, 5389−5392.
Stahl, N., & Jencks, W. P. (1987) *Biochemistry 26*, 7654−7667.
Taniguchi, K., Suzuki, K., Kai, D., Matsuoka, I., Tomita, K., & Iida, S. (1984) *J. Biol. Chem. 259*, 15228−15233.
Taniguchi, K., Suzuki, K., Sasaki, T., Shimokobe, H., & Iida, S. (1986) *J. Biochem. 100*, 1231−1239.
Taniguchi, K., Sasaki, T., Shinoguchi, E., Kamo, Y., & Ito, E. (1991) *J. Biochem. 109*, 299−306.
Verjovski-Almeida, S., Kurzmack, M., & Inesi, G. (1978) *Biochemistry 17*, 5006−5013.

BI961324W