Inhibition of Sodium and Potassium Adenosine Triphosphatase by \(2',3'O-(2,4,6\text{-Trinitrocylohexadienylidene})\) Adenine Nucleotides

IMPLICATIONS FOR THE STRUCTURE AND MECHANISM OF THE \(\text{Na}^+\text{K}^+\) PUMP*

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Trinitrophenyl derivatives of adenine nucleotides (TNP-nucleotides: \(2',3'O-2,4,6\text{-trinitrocylohexadienylidene}\) complexes at neutral or basic pH) are potent inhibitors of (Na,K)-ATPase activity. The inhibitory potency of the derivatives tested followed the sequence: TNP-ADP > TNP-ATP > TNP-AMP > TNP-IMP > TNP-adenosine. In the presence of \(\text{Na}^+\) plus \(K^+\), high and low affinity activation of ATPase activity by ATP was observed. Under these conditions, TNP-ATP inhibited (Na,K)-ATPase activity competitively with respect to ATP at the kinetically defined "low affinity ATP site." In the presence of \(\text{Na}^+\) alone, only high affinity activation by ATP was observed. Under these conditions, TNP-ATP inhibited (Na)-ATPase and enzyme phosphorylation by competing with ATP at the kinetically defined "high affinity ATP site." The \(K_i\) values for inhibition were similar to the \(K_v\) values determined by direct TNP-ATP binding measurements, indicating that the same TNP-ATP site is involved in the inhibition of (Na,K)-ATPase and (Na)-ATPase activities. We conclude that high and low affinity ATP "sites" are interconvertible (i.e. they represent two forms of the same site) and do not co-exist independently. TNP-ATP also inhibited competitively the \(K^+\)-stimulated \(p\)-nitrophenyl phosphatase activity and enzyme phosphorylation by \(P_i\), suggesting that the catalytic site for these substrates is associated with the TNP-ATP site. A kinetic model for (Na,K)-ATPase turnover based on a single ATP site which changes affinity during turnover is presented. The model was analyzed by the King-Altman (1956) \textit{J. Phys. Chem.} 60, 1375-1378 method to obtain the steady state equation for the rate of ATP hydrolysis as a function of ATP concentration. Computer simulations using published values of the rate constants of intermediate steps suggest that the model is adequate to describe the observed dependence of enzyme activity on ATP concentration and the inhibition by TNP-ATP. The implications of these results on the structure and mechanism of the (Na,K) pump are discussed.

\((\text{Na,K})\text{-ATPase}\) exhibits complex kinetics of activation with respect to ATP. In the presence of \(\text{Na}^+\) and \(K^+\), double reciprocal plots of ATPase velocity \textit{versus} ATP concentration are convex upward (1-6). By extrapolating linear regions, two apparent \(K_v\) values for ATP and two apparent \(V_{max}\) values for the limiting cases ([ATP] \(\rightarrow 0\) and [ATP] \(\rightarrow \infty\)) are obtained. The high and low affinity \(K_v\) values lie in the ranges of 0.1-80 \(\mu M\) and 0.1 to 2 \(mM\) depending on the source of the enzyme, the temperature of the assay, and the concentrations of \(\text{Na}^+\), \(K^+\), and \(Mg^{2+}\). This behavior has been interpreted by postulating the existence of two independent nucleotide binding sites or as negative cooperativity between two identical sites on separate subunits of a functional dimeric complex (3, 6, 7).

In contrast to the complex kinetic behavior in the presence of \(\text{Na}^+\) and \(K^+\), all (Na,K)-ATPase preparations tested exhibit a \(\text{Na}^+\)-dependent ATPase activity which follows classical Michaelis-Menten kinetics with a high affinity \(K_v\) (0.1-1 \(\mu M\)) for ATP (1, 3, 8). The initial rate and equilibrium level of \(\text{Na}^+\)-dependent enzyme phosphorylation by \(\gamma^{32}P\)ATP and the initial rate of ouabain binding in the presence of \(\text{Na}^+\) also exhibit simple high affinity activation by ATP (5, 9, 10). This (Na)-ATPase activity corresponds to a \(\text{Na}^+\) efflux in resealed erythrocyte ghosts which is saturated at 1 \(\mu M\) internal ATP in the absence of external \(\text{Na}^+\) and \(K^+\) (4).

The low affinity activation by ATP is observed in the presence of \(K^+\). Post et al. (11) showed that ATP stimulated rephosphorylation of enzyme dephosphorylated in the presence of \(K^+\). These studies suggested that a form of the enzyme which contained bound \(K^+\), \(E_2(K)\), is slowly converted back to a form of the enzyme (\(E_1\)) that can be rephosphorylated, and that ATP binding to a low affinity site is required to release the bound \(K^+\). This hypothesis received support from studies of the effect of \(\text{Na}^+\) and \(K^+\) on hydrolysis by trypsin (12, 13), formycin triphosphate binding (14, 15), and tryptophan (16) or fluorescein (17) fluorescence, all of which showed that the enzyme exists in distinct conformations in the presence of ATP.

The abbreviations used are: (Na,K)-ATPase, sodium-potassium transport adenosine triphosphatase; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)N,N,N',N'-tetraacetic acid; ECTA, ethylene glycol bis([\(\beta\]-aminophenyl ether)]N,N,N',N'-tetraacetic acid; pNPP, p-nitrophenyl phosphate; TNP-X, 2',3'O-(2,4,6-trinitrocylohexadienylidene) complexes of nucleotides or nucleosides at neutral or basic pH, where X is adenosine, AMP, ADP, ATP, or IMP, at acid pH the TNP moiety is 2'(or 3')-O-(2,4,6-trinitrophenyl). The ribose hydroxyl of the complex has a \(pK_v\) of 5.1; AMP-PNP, adenylyl-5'-yl imidodiphosphate; AMP-PCP, adenylyl-5'-yl (\(\beta\),\(\gamma\)-methylene) diphosphate.

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ence of either ion and that ATP is able to accelerate the slow conversion of the K form to the Na form. Recently, direct evidence for $E_2(K)$ has been reported (18).

Additional evidence for low affinity activation by ATP is that $K^-K^+$ exchange in erythrocyte ghosts occurs without ATP hydrolysis and requires high concentrations of ATP or a nonhydrolyzable ATP analogue (19, 20). This transport activity appears to be associated with reversible enzyme phosphorylation from Pi in the presence of $K^+$ as evidenced by a single ATP site is presented. A preliminary communication of site undergoes changes in affinity for ATP during turnover. A and other TNP nucleotides. The results suggest that a single kinetic studies of (Na,K)-ATPase inhibition by TNP-ATP and low affinity capacities has been well studied, the structural identity of the ATP sites is not known. In the preceding paper (22), evidence is presented suggesting that TNP-ATP binds to a single ATP site on (Na,K)-ATPase. This paper presents kinetic studies of (Na,K)-ATPase inhibition by TNP-ATP and other TNP nucleotides. The results suggest that a single site undergoes changes in affinity for ATP during turnover. A kinetic mechanism for (Na,K)-ATPase turnover based on a single ATP site is presented. A preliminary communication of parts of this work has been presented (23).

EXPERIMENTAL PROCEDURES

Eel electroplax (Na,K)-ATPase was prepared as described previously (22, 24). Except where indicated, protein was determined by a modification (25) of the method of Lowry et al. (26), without corrections. This method overestimates the (Na,K)-ATPase protein by 28% when compared with quantitative amino acid analysis (22), giving lower estimates of specific activity. ATPase activity was measured by assay of inorganic phosphate in butyl acetate extracts of the incubation medium according to the method of Samui (27). Phosphate concentration was determined by absorbance at 310 nm or by liquid scintillation counting in experiments at low ATP concentration (Figs. 2 and 4) where $[^{32}P]ATP$ was used.

The released phosphate was measured after 1- to 5-min incubations in a total volume of 1 ml of various reaction mixtures given in the figure legends. The reaction was terminated by addition of 1 ml of cold 16% trichloroacetic acid. Blanks for the phosphate assay consisted of reaction mixtures containing ATP without enzyme treated identically with the samples containing the enzyme. The blank minus enzyme was equivalent to blanks containing enzyme plus 0.1 mM ouabain or blanks containing 100 mM KCl and no NaCl. (Na,K)-ATPase activity was also measured at constant ATP concentrations by a coupled enzyme assay in which the decrease in NADH absorbance at 340 nm ($e_{340} = 6.2 \times 10^3$ M$^{-1}$ cm$^{-1}$) was measured continuously in cuvettes containing 0.1 mM NADH, 4 mM phosphoenolpyruvate, 50 $\mu$M of pyruvate kinase (515 units/mg), and 30 $\mu$M of lactate dehydrogenase (860 units/mg). One $\mu$M TNP-ATP had no measurable effect on the activity of the pyruvate kinase-lactate dehydrogenase ATP-regenerating system as assayed by the addition of ADP. K$^+$-phosphatase activity was measured continuously by following the increase in absorbance at 410 nm ($e_{410} = 1.6 \times 10^5$ M$^{-1}$ cm$^{-1}$) due to release of $p$-nitrophenolate anion from $p$-nitrophenyl phosphate. ATPase activity was linear with time in the absence or presence of TNP-ATP and the activity was also a linear function of (Na,K)-ATPase concentration. For initial rate measurements of ATPase activity or K$^+$-phosphatase activity as a function of substrate concentration, the conditions were such that not more than 10% of the substrate was hydrolyzed over the measured time course.

ATP, AMP-PCP, and AMP-PNP were purchased from Boehringer Mannheim. NADH, phosphoenolpyruvate, $p$-nitrophenyl phosphate, lactate dehydrogenase, and pyruvate kinase were from Sigma. TNP-ATP, TNP-ADP, TNP-AMP, TNP-IMP, and TNP-adenosine were obtained from Molecular Probes of Plano, TX. $[^{32}P]ATP$ was from ICN.

RESULTS

Inhibitory Properties of TNP Nucleotides—Although TNP-ATP is a substrate of myosin (28), it was not hydrolyzed by (Na,K)-ATPase. There was no detectable release of P$\gamma$S from TNP-ATP when 0.62 mM TNP-ATP was incubated with 32 $\mu$g/ml of eel (Na,K)-ATPase at 37°C for 1 h in the presence of 40 mM Tris-HCl, pH 7.5, 4.7 mM MgCl$\text{2}^-$, and either 120 mM NaCl, 20 mM KCl, or 120 mM NaCl plus 20 mM KCl. Based on the limit of detectability of inorganic phosphate by the assay used (27), the maximum rate of TNP-ATP hydrolysis by (Na,K)-ATPase was no higher than 3 x 10$^{-5}$-fold that of ATP hydrolysis.

TNP-ATP is a potent inhibitor of (Na,K)-ATPase. In order to investigate the specificity of inhibition, we tested the effect of several trinitrocyclohexadienyl derivatives on (Na,K)-ATPase activity in the presence of 3 mM ATP. The dose-response curves in Fig. 1 show that the most potent inhibitors were TNP-ADP and TNP-ATP. The inhibitory potency decreased in the sequence: TNP-ADP > TNP-ATP > TNP-IMP > TNP-adenosine. Trinitrophenol did not inhibit at concentrations up to 1 mM. The difference in inhibitory potency of the various derivatives indicates that high affinity inhibition requires the presence of the adenine base and both $\alpha$ and $\beta$ phosphates in addition to the trinitrocyclohexadienyl moiety.

TNP-ATP decreased the rate of ATP hydrolysis immediately upon addition and the remaining activity was linear with time. No evidence of time-dependent inhibition was obtained by P$\gamma$S assay, p-histidine assay, or coupled enzyme assay of (Na,K)-ATPase activity. To test for reversibility of inhibition, the enzyme was preincubated with 10 $\mu$M TNP-ADP in the presence or absence of Na$^+$, K$^+$, and Mg$^{2+}$. This concentration of TNP-ADP was sufficient to inhibit 75% of the activity in the

![Fig. 1. Effect of TNP nucleotides on (Na,K)-ATPase activity. Eel (Na,K)-ATPase (4-12 $\mu$g/ml) was incubated for 5 min at 37°C in 30 mM histidine/HCl, pH 7.5, 120 mM NaCl, 20 mM KCl, 4 mM MgCl$\text{2}^-$, 1 mM EDTA, 3 mM N$\text{a}_2$EDTA, and the indicated concentrations of the inhibitors. The ATPase activity is expressed relative to the activity in the absence of inhibitor.](image-url)
presence of 3 mM ATP. After 10 min at 23°C, the suspensions were diluted 100-fold and (Na,K)-ATPase activity was measured under the conditions of Fig. 1. More than 95% of the activity was regained after dilution when compared to enzyme that had not been exposed to TNP-ADP regardless of which ions were present in the preincubation (not shown). Thus, the inhibition of (Na,K)-ATPase activity by TNP nucleotides appears to be immediate and fully reversible.

Kinetic Analysis of (Na,K)-ATPase Inhibition by TNP-ATP—The inhibition of (Na,K)-ATPase activity by the TNP nucleotides was more powerful than that observed with other nonhydrolyzable ATP analogues. Whereas 3 μM TNP-ADP and 5 μM TNP-ATP inhibited (Na,K)-ATPase 50% in the presence of about a 1000-fold excess of ATP (Fig. 1), less than 5% inhibition was observed with up to 100 μM concentration of either the β,γ imido or β,γ methylene ATP analogues under the same conditions (not shown).

Fig. 2 shows double reciprocal plots of (Na,K)-ATPase activity in the range of high affinity activation by ATP (0.5–3 μM). At these low ATP concentrations (apparent K, = 3.6 μM), TNP-ATP inhibited mainly by lowering Vmax, although there appears to be also a small increase in the apparent K,.

The K, for TNP-ATP derived from a Dixon plot of the data was about 0.12 μM. The noncompetitive or mixed inhibition by TNP-ATP under these conditions suggests that TNP-ATP interacts at a site other than the high affinity ATP site. This interpretation, however, is not unique. Apparent noncompetitive inhibition could result if TNP-ATP were able to bind to the high affinity ATP site at a step subsequent to phosphorylation, if the affinity for ATP decreased while that for TNP-ATP remained high, as discussed below.

Fig. 3 shows double reciprocal plots of (Na,K)-ATPase activity in the range of low affinity activation by ATP (0.1–5 mM). In this range of ATP concentration, TNP-ATP inhibition was strictly competitive with ATP (Fig. 3). The derived K, for TNP-ATP was 0.36 ± 0.08 μM at 37°C (Fig. 3) and 0.07 μM at 25°C.

When ATPase activity was measured with Na* alone, in the absence of K+, TNP-ATP produced different behavior. No inhibition of (Na)-ATPase activity in the presence of 3 mM ATP was observed with 10 μM TNP-ADP or TNP-ATP, whereas this concentration inhibited (Na,K)-ATPase activity 75% or 65%, respectively. At low ATP concentrations, TNP-ATP inhibited competitively with respect to ATP. Fig. 4 shows double reciprocal plots of (Na)-ATPase activity, which exhibited only high affinity activation by ATP (K, = 0.42 μM). The derived K, for TNP-ATP was 0.11 ± 0.01 μM at 37°C.

TNP-ATP Inhibition of p-Nitrophenyl Phosphatase Activity and Phosphorylation—TNP behaves as a classical competitive inhibitor of p-NPPase activity in the absence of Na* and presence of nonlimiting K+ (3, 29). It has been suggested that the binding site for p-NPP is identical with the "low" affinity ATP site (3). However, the affinity with which ATP inhibits p-NPPase activity is strongly dependent on K+ concentration (29, 30). In one study, the K, for ATP increased from about 1 μM at 0.6 mM K+ to about 0.6 mM at 100 mM K+ (29). Furthermore, it has been suggested that the p-NPP binding site is different from the high affinity ATP site on the basis of different sensitivities of the ATPase and p-NPPase activities to limited proteolysis by trypsin (13). Since TNP-ATP binding is less sensitive to K+ than ATP binding (22), it was of interest to study the effect of TNP-ATP on p-NPPase activity as a function of K+.

Fig. 5 shows double reciprocal plots of p-NPPase activity as a function of p-NPP concentration in the presence of either 20 mM K+ or 150 mM K+. At 20 mM K+, TNP-ATP inhibited competitively with p-NPP (Fig. 5A). Dixon plots of these experiments were linear up to about 0.3 μM TNP-ATP, but became convex upward at higher concentrations (not shown). Similar nonlinear Dixon plots were obtained when ATP was used instead of TNP-ATP (not shown). This apparent deviation from simple competitive behavior may result from decreased affinity for K+ in the presence of TNP-ATP, so that both p-NPP and K+ become rate limiting at the higher TNP-ATP concentrations, as previously shown for ATP (30). Indeed, when K+ was increased to 150 mM, the apparent K, increased linearly with TNP-ATP over a wider range of inhibitor concentration, probably because K+ was still near saturation even with 6.2 μM TNP-ATP (Fig. 5B). Under these conditions, the curvature in the Dixon plots was less significant. The derived K, for TNP-ATP was 0.1–0.2 μM at 20 mM K+ and 1.2 μM at 150 mM K+.

FIG. 2. Effect of TNP-ATP on (Na,K)-ATPase activity at low ATP concentration. (Na,K)-ATPase (0.2 μg/ml) was incubated 1 min at 37°C in 45 mM Tris-HCl, pH 7.5, 108 mM NaCl, 18 mM KCl, 1.4 mM MgCl2, 0.4 mM EDTA, and the indicated concentrations of ATP and TNP-ATP. ATPase activity was measured by assay of 32P released from γ-[32P]ATP (3.6 × 106 cpm/mmol).

FIG. 3. Effect of TNP-ATP on (Na,K)-ATPase activity at high ATP concentration. P, production was assayed after 5-min incubation under the following conditions: 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM KCl, 5 mM MgCl2, 1 mM EGTA, and 2 μg/ml of (Na,K)-ATPase. Temperature = 37°C. The inset shows the apparent K, as a function of TNP-ATP concentration.

FIG. 5. Effect of TNP-ATP on p-NPPase activity at low K+ concentration. p-NPP (0.2 μg/ml) was incubated 1 min at 37°C in 45 mM Tris-HCl, pH 7.5, 108 mM NaCl, 18 mM KCl, 1.4 mM MgCl2, 0.4 mM EDTA, and the indicated concentrations of ATP and TNP-ATP. P, production was assayed after 5-min incubation under the following conditions: 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM KCl, 5 mM MgCl2, 1 mM EGTA, and 2 μg/ml of (Na,K)-ATPase. Temperature = 37°C. The inset shows the apparent K, as a function of TNP-ATP concentration.
Dissociation constants for TNP nucleotide inhibition of (Na,K)-ATPase phosphorylation by ATP or P, at 0°C.

Data kindly provided by Dr. Robert L. Post, Vanderbilt University School of Medicine.

| Inhibitor | \( K_T \) (Na') form | \( K_T \) (K') form |
|-----------|------------------------|---------------------|
| TNP-AMP   | 0.093 \( \mu M \)      | 8.0 \( \mu M \)     |
| TNP-ADP   | 0.043 \( \mu M \)      | 1.0 \( \mu M \)     |
| TNP-ATP   | 0.063 \( \mu M \)      | 1.5 \( \mu M \)     |
| ATP       | 0.16 \( \mu M \)       | 32 \( \mu M \)      |
|           | 200 \( \mu M \)        |                     |

* The incubation medium contained 20 mM imidazole-Taps, pH 7.8, 32 mM NaCl, and 10 mM \( \gamma^32P\)ATP. Phosphorylation was only by bound ATP using a transient Mg\(^2+\) pulse.
* The incubation medium contained 20 mM imidazole glycyl-glycine, pH 7.5, 100 mM K\(^+\) acetate, 0.2 mM (Tris), EDTA, and 0.6 mM \( \gamma^32P\). Phosphorylation was started by addition of 1 mM MgCl\(_2\) and stopped with acid after 10 s.
* \( K_{P} \) for ATP.

Additional information on the inhibitory effects of the TNP nucleotides arises from phosphorylation experiments carried out by Dr. Robert L. Post at the Department of Physiology, Vanderbilt University School of Medicine. At 0°C, TNP-AMP, TNP-ADP, and TNP-ATP inhibited phosphorylation of (Na,K)-ATPase by ATP in the presence of 32 mM Na\(^+\) or by P, in the presence of 100 mM K\(^+\). The \( K_T \) values derived from the experiments, assuming simple competitive inhibition, are shown in Table I. These results follow the same pattern seen with (Na,K)-ATPase and phosphatase activities. The difference in affinity of the Na\(^+\) and K\(^+\) forms of the enzyme for the TNP nucleotides was much smaller than for ATP. In the presence of K\(^+\), the \( K_{0.5} \) for ATP inhibition of phosphorylation by P, was 32 \( \mu M \), whereas the \( K_{P} \) for ATP binding in the presence of Na\(^+\) was 0.16 \( \mu M \). In contrast, the \( K_{P} \) for TNP-ATP only increased from 0.063 \( \mu M \) in the presence of Na\(^+\) to 1.5 \( \mu M \) in the presence of K\(^+\) (Table I). The inhibitory effect of TNP-AMP is particularly striking, since its \( K_T \) was 0.093 \( \mu M \) in the presence of Na\(^+\), while the estimated \( K_{P} \) for AMP binding to a similar (Na,K)-ATPase preparation was 600 \( \mu M \) (31), a difference of about 6500-fold. In addition, the ouabain-complexed enzyme appears to retain high affinity for TNP-ADP and TNP-ATP. Phosphorylation by P, in the presence of 0.25 mM ouabain and the absence of K\(^+\) was inhibited 39% by 4 \( \mu M \) TNP-ADP and fluorometric studies showed that TNP-ATP binds in the presence of 1 mM ouabain with a \( K_{P} \) of about 1.2 \( \mu M \). In contrast, ATP does not appear to bind in the presence of ouabain (32).

A Kinetic Model for (Na,K)-ATPase Turnover Based on a Single ATP Site—Based on the binding and inhibition properties of TNP-ATP, we propose that the functional enzyme has a single ATP binding site which changes conformation from high to low affinity during turnover.

Fig. 6 shows the proposed kinetic model in terms of the major enzyme species that exist in the presence of optimal Na\(^+\), K\(^+\), and Mg\(^2+\) concentrations and the pathways of their interconversion. The nomenclature of the different intermediates and the reaction sequence are essentially the same as that of the Post-Albers scheme (33). The \( E_1 \) and \( E_2 \) conformations are assumed to contain bound Na\(^+\) and K\(^+\), respectively. For simplicity, the ion binding and dissociation steps are omitted and Mg\(^2+\) is assumed to be bound to the enzyme at all times. The evidence for this reaction sequence and the role of the intermediate steps in ion translocation are discussed by Post (33) and Karlish et al. (15). The model in Fig. 6 assumes that the functional ion translocating complex has a single ATP site. This site can exist in two conformations and

\[^2\]P. A. G. Fortes and E. G. Moczydlowski, unpublished observations.
FIG. 6. A kinetic model for (Na,K)-ATPase turnover based on a single ATP site which exists in two conformations, E₁ and E₂. See text for discussion.

Fig. 7. Comparison of a double reciprocal plot obtained by computer simulation of the rate dependence of (Na,K)-ATPase activity on ATP concentration with actual data for eel (Na,K)-ATPase. In A, the turnover number, v/E₉, was calculated as a function of ATP concentration according to Equation 3 in the Appendix and the values for the coefficients and rate constants listed in Tables IA and IIA, where [I] = 0. The numerical values for the coefficients of Equation 3 were: a = 8.43 × 10⁻³⁷ M⁻² min⁻¹, b = 2.49 × 10⁻³⁴ M⁻² min⁻¹, c = 5.91 × 10⁻²⁹ M⁻² min⁻¹, d = 1.52 × 10⁻³⁵ M⁻² min⁻¹, and e = 1.06 × 10⁻²² min⁻². The solid curve is the calculated double reciprocal plot and the dotted lines are the asymptotes of the limiting regions of the curve based on Equations 4 and 5. In A, (Na,K)-ATPase activity was measured at constant [ATP] by the coupled enzyme assay. The specific activity is based on protein measured by amino acid analysis and the turnover number is given per nucleotide site based on TNP-ATP titrations (22). Conditions: 50 mM histidine/HCl, pH 7.5, 130 mM NaCl, 20 mM KC1, 5 mM MgCl₂, and 0.2 or 0.4 µg/ml of (Na,K)-ATPase. Temperature 25°C.

plays a dual functional role. In the E₁ conformation, the site has high affinity for ATP and participates in Na⁺-dependent phosphorylation. In the E₂ conformation, the ATP site has low affinity for ATP, but ATP binding promotes K⁺ dissociation, accelerating conversion to the high affinity form E₁-ATP, which increases the turnover rate.

If this proposal is to be a tenable alternative to two-site models (6, 7), it must be shown that a one-site model can account for the complex kinetics of ATP activation of the enzyme. Fig. 7A shows a computer-generated double reciprocal plot of (Na,K)-ATPase activity as a function of [ATP] using the rate constants in Table IIA and the steady state rate equation for the model in Fig. 6 as derived in the appendix. The theoretical curve approaches the asymptotes shown as dotted lines in Fig. 7A in the limits of zero and infinite [ATP]. The asymptotes are defined by the following parameters: (Equations 4 and 5): Kₘ₁ = 0.9 µM; Vₘ₅₅ = 21.4 min⁻¹; Kₘ₂ = 254 µM; and Vₘ₅₅ = 1427 min⁻¹. It is important to note that at 0.5 µM ATP, the curve is still far from its asymptote (dashed line in Fig. 7A). If these were experimental data, a tangent to the curve in this region would give higher Kₘ₁ and Vₘ₅₅ values of these parameters. The simulated curve resembles the observed behavior (Fig. 7B). No attempt was made to adjust the kinetic parameters to fit actual data since a
These predictions are in excellent agreement with the experiments, which show a slight increase in concentration simulations of dephosphorylation rates in the eel enzyme in Tables IA and IIA to solve Equations TNP-ATP concentration was investigated using the constants are increased 10- to 100-fold in the range used in recent TNP-ATP does not affect activity at low and high ATP concentration are shown in Fig. apparent K, values derived from the experimental and simulated curves are similar. But the sim- The estimated turnover number per nucleotide site was 4,470 min⁻¹. It is evident that the apparent Km values derived from the experimental and simulated curves are similar. But the simulation underestimates the turnover number and the relative rates of the high and low affinity regions. These parameters are sensitive to the values of several constants, particularly k1 and k6, the rates of E1 → E2 conversion, which have not been measured for the eel enzyme. If the values of these constants are increased 10- to 100-fold in the range used in recent simulations of dephosphorylation rates in the eel enzyme (34), better fits to the experimental curve are obtained.

The simulated effects of TNP-ATP on (Na,K)-ATPase activity at low and high ATP concentration are shown in Fig. 8, A and B, respectively. Analytically, it can be shown that TNP-ATP does not affect Vmax since Vmax is only a function of the terms a and c (Equation 5) that do not contain the TNP-ATP-dependent terms. Kni, Vmax and Kni are affected by TNP-ATP since they depend on either d or both d and e (Equations 4 and 5). The variation of Kni, Vmax, and Kni on TNP-ATP concentration was investigated using the constants in Tables IA and IIA to solve Equations 4 and 5. The results indicated that Kni is relatively insensitive to TNP-ATP concentration (Kni increases 9% with 1 μM TNP-ATP), while Kni and (Vmax)⁻¹ are linear functions of (1 + [I]/Kd) (not shown). These predictions are in excellent agreement with the experiments, which show a slight increase in Kni, but essentially noncompetitive inhibition by TNP-ATP at low ATP concentration (Fig. 2) and strictly competitive inhibition at high ATP concentration (Fig. 3).

### DISCUSSION

In the accompanying paper, we showed that TNP-ATP binds to a single site per ouabain site. The (Na,K)-ATPase site was identified with the ATP site of the enzyme on the basis of binding competition between ATP and TNP-ATP (22). The present results show that TNP-ATP inhibited ATPase activity (Figs. 3 and 4) and enzyme phosphorylation (Table I) in a competitive fashion with respect to ATP, as would be expected from the binding studies. TNP-ATP inhibition of phosphatase activity (Fig. 5) and phosphorylation by P, (Table I) also appear to be competitive with substrate. This suggests that the substrate site for each of these reactions is equivalent to or associated with the TNP-ATP binding site. The K values for TNP-ATP derived from the inhibition studies were in the same range (0.06-1.5 μM) as the Kd obtained from equilibrium TNP-ATP binding (6.04-0.7 μM), and both exhibited a similar sensitivity to K⁺. The larger K values were obtained in the presence of high K⁺ (Figs. 3 and 5 and Table I), and the lower K values were obtained in the presence of Na⁺ alone (Fig. 4 and Table I), in agreement with the increase in Kd for TNP-ATP in the presence of K⁺ measured by equilibrium binding (22). These observations indicate that occupation of the same TNP-ATP site measured by equilibrium binding is responsible for inhibition.

A comparison of TNP-ATP inhibition of (Na⁺)-ATPase and (Na⁺ + K⁺) ATPase activities is of particular interest. In the presence of Na⁺ alone, TNP-ATP inhibited by competing with ATP at the kinetically defined "high affinity site" (Fig.

### TABLE I

| Coefficients of the rate equation (Equations 2 and 3) of a proposed kinetic model for (Na,K)-ATPase turnover (Fig. 8) as solved by the King Altman method |
|---|
| a = k₄k₈k₁₃ / K₅s₂ |
| b = k₃k₇k₉ (k₁ / K₅s₁K₁₂ + k₂k₁₃ / K₅s₂K₄) |
| c = 1 / K₅s₂ (k₃k₇k₁₃ / K₅s₁K₁₂ + k₃k₇k₁₃ / K₅s₂K₄ + k₃k₇k₁₃ / K₅s₂K₄) |
| d = (1 + [I]/K₁₂) / (K₅s₁ + k₅k₇k₁₃ / K₅s₂K₄) |

unique fit is not possible given the number of parameters. Nevertheless, a comparison between theoretical and experimental curves is of interest. The experimental curve (lowest [ATP] tested was 1.6 μM) gives Kmax = 2 μM, Vmax = 2.3 μmol min⁻¹·mg⁻¹, Kd = 170 μM, and Vd = 26 μmol min⁻¹·mg⁻¹. The estimated turnover number per nucleotide site (M = 175,000, Ref. 22) was 4,470 min⁻¹.
we propose that (Na,K)-ATPase has a single ATP site which decreases its affinity for ATP after phosphorylation when K+ or ouabain bind to the enzyme. TNP-ATP also binds to this site but the decrease in its affinity caused by K+ or ouabain is smaller than for ATP (Table I and Ref. 22). This predicts that: (i) in the presence of Na+ alone, simple competition of TNP-ATP with ATP binding at a high affinity site should occur, as was observed by Na+-ATPase (Fig. 4), phosphorylation (Table I), and direct binding (22) measurements; (ii) in the presence of Na+ and K+, ATP should displace TNP-ATP with high affinity before phosphorylation, but once phosphorylation occurred, K+ would bind and the affinity for ATP would decrease, whereas that for TNP-ATP would remain high so that only high ATP concentrations could displace TNP-ATP. This should result in noncompetitive inhibition of (Na+ + K+) ATPase activity at low [ATP] as was observed in Fig. 2 or competitive inhibition at high [ATP] as was observed in Fig. 3.

In order to provide a quantitative framework to evaluate the above hypothesis, we analyzed a kinetic model for (Na,K)-ATPase turnover based on a single ATP site (Fig. 9). Computer simulations using the single site model gave nonlinear double reciprocal plots of (Na,K)-ATPase activity versus [ATP] (Fig. 7A), similar to those obtained experimentally (Fig. 7B). The simulation of the effects of TNP-ATP predicted that inhibition of (Na,K)-ATPase should appear noncompetitive at low [ATP] (Fig. 8A) and competitive at high [ATP] (Fig. 8B), as was actually observed (Figs. 2 and 3). At present, uncertainties in the values of several of the rate constants do not permit a rigorous quantitative comparison of the model with actual initial rate data. This must await experimental information on the values of the rate constants measured in the same enzyme and under the same experimental conditions. Nevertheless, the single site model presented here accommodates the observed properties of the enzyme and may provide a useful framework for further studies of the mechanism of (Na,K)-ATPase.

Based on the single site model, a possible conformational basis of the association between the changes in ligand affinity and ion transport is shown schematically in Fig. 9. The ions are transported through a channel that may be formed by the α chain alone or at the interface between α and β subunits as in the transport models proposed by Jardetzky (35) and Själer (36). ATP and ouabain are assumed to bind to or near the internal and external portions of the channel, respectively. Conformational changes that accompany ion binding, phosphorylation, dephosphorylation, and ion release cause reciprocal changes in affinity for ATP and ouabain. When the affinity for ATP is high, that for ouabain is low, and vice versa, except when K+ is bound (E2(K)), which causes low affinity for both ATP and ouabain. Although the distance between internal and external sites appears to be about 60 to 70 Å (24), small conformational changes causing increased or decreased ouabain and ATP affinity could be transmitted across the membrane if the protein segments crossing the bilayer are rigid. This is represented schematically in Fig. 9 where the "opening" (i.e. the high affinity form) of the ligand site on one side of the membrane is accompanied by the "closing" (i.e. the low affinity form) of the site at the opposite side. During turnover, ATP binds with high affinity to the E1 form; if Na+ is present, 3 Na+ ions enter the channel, the γ phosphate is transferred to the protein, and the enzyme changes to a new form in which the internal site decreases in affinity, causing release of ADP (E1 ~ P(Na)). This form is unstable and relaxes rapidly to the E1 ~ P form in which the ATP affinity is further decreased, and the ouabain affinity increases. During the E1 ~ P ~ E2 ~ P transition, Na+ is released. Either E1 ~ P or E2 ~ P can bind ouabain with high

### Table I

| Constant | Value used | Reference |
|----------|------------|-----------|
| k_1 | 14 min⁻¹ | (15, 16) |
| k_2 | 500 min⁻¹ | Footnote a |
| k_3 | 540 min⁻¹ | (59) |
| k_4 | 11,000 min⁻¹ | (60) |
| k_5 | 4,600 min⁻¹ | (60) |
| k_6 | 15,000 min⁻¹ | (60) |
| k_7 | 50,000 min⁻¹ | Footnote b |
| k_8 | 3,240 min⁻¹ | (16), Footnote c |
| k_9 | 1,000 min⁻¹ | (15), Footnote d |
| k_10 | 7 × 10⁻⁴ M | (22) |
| k_11 | 5 × 10⁻⁴ M | (16) |
| k_12 | This paper |
| k_13 | 4 × 10⁻⁴ M | This paper |

* The value of k_2 could be determined from the time course of the increase in tryptophan fluorescence observed upon adding K+ to E1. Karlish and Yates (16) did not report a value for this rate constant due to technical difficulties. As k_2 is increased from 500 to 5000 min⁻¹ with the other constants held fixed, k_4, k_5, and V_4,5 are unaffected while k_2, k_1, and V_1,2 are increased. Here a value of 500 min⁻¹ is assumed.

* Although k_2 has not been measured, we may estimate its value from the relationship k_2 = k_2h_1. We obtained a value of about 10⁻⁴ M⁻¹ for the k_0 of ATP in the presence of K+ and Mg2⁺ at 3°C. This value is probably a lower limit of the value at 22°C since ATP may exhibit a negative enthalpy of binding to the E_1 form of (Na,K)-ATPase (2). Here k_0 is given the value of 5 × 10⁻⁴ M⁻¹ at 22°C estimated in Ref. 16. It may be assumed that k_1h has a value near 10⁻⁸ M⁻¹ min⁻¹, which is a typical rate for fast associating ligands to proteins (Ref. 61, pp. 130–131). These estimates give a value of 5 × 10⁻⁴ M⁻¹ min⁻¹ for k_0 which is used here. The effect of varying this rate constant from 10⁻⁸ to 10⁻⁷ with the other constants held fixed was tested. As k_2 is increased, k_1, and V_1,2 are increased, k_0 is decreased, and V_0,1,2 is unaffected.

* Karlish and Yates (16) have measured the rate of the E_1 ~ E_2 ATP transition in the presence of various concentrations of ATP by monitoring the change in enzyme tryptophan fluorescence upon adding Na+ and ATP to enzyme incubated with K+. They estimated a rate of 3240 min⁻¹ at saturating ATP concentrations. This transition is assumed to occur via the k_11, k_12 pathway since the rate is much faster than the E_1 ~ E_2 transition. Since the rate of ATP binding is likely to be faster than the E_2-ATP ~ E_2-ATP transition, this value is assumed to be a good approximation of k_11.

* Karlish et al. (15) measured the release of formycin diphosphate bound to E1 by monitoring the fluorescence decrease observed upon the addition of K+. This transition is assumed to represent internal sites are rigid. This is represented schematically in Fig. 9 where the "opening" (i.e. the high affinity form) of the ligand site on one side of the membrane is accompanied by the "closing" (i.e. the low affinity form) of the site at the opposite side. During turnover, ATP binds with high affinity to the E1 form; if Na+ is present, 3 Na+ ions enter the channel, the γ phosphate is transferred to the protein, and the enzyme changes to a new form in which the internal site decreases in affinity, causing release of ADP (E1 ~ P(Na)). This form is unstable and relaxes rapidly to the E1 ~ P form in which the ATP affinity is further decreased, and the ouabain affinity increases. During the E1 ~ P ~ E2 ~ P transition, Na+ is released. Either E1 ~ P or E2 ~ P can bind ouabain with high
The present results and conclusions are in conflict with recent models of (Na,K)-ATPase structure and mechanism. It has been proposed that the functional (Na,K)-ATPase is an oligomer, containing two large (α) and two small (β) chains, which exhibits half-of-sites reactivity toward phosphorylation and binding of ouabain or ATP (6, 12, 63). It has also been proposed that distinct high and low affinity sites for ATP are present at all times in the enzyme, and catalysis proceeds by a flip-flop mechanism involving alternate phosphorylation of the individual α subunits of the oligomer (3, 6, 7, 63). However, the evidence on which the above models are based is subject to alternative interpretations. TNP-ATP and ouabain binding studies (22) have suggested that each αβ protomer contains one nucleotide and one ouabain binding site, in conflict with the notion that (Na,K)-ATPase exhibits half-of-sites behavior. Furthermore, ATP binding measured by competition with TNP-ATP at low temperature did not produce any evidence of independently co-existent high and low affinity ATP sites (22). A previous study of vanadate binding and inhibition of (Na,K)-ATPase reported that there is one high and one low affinity vanadate site per ouabain site (6). Although the authors interpreted these vanadate sites as corresponding to the "low" and "high" affinity ATP sites, respectively, an alternative interpretation is that only the high affinity vanadate site interacts with the ATP site, causing effects similar to those of TNP-ATP, and the low affinity vanadate site has no functional significance. Additional arguments for the existence of distinct high and low affinity ATP sites have been based on the interpretation of (Na,K)-ATPase kinetics (3, 6, 7, 44, 63) and the kinetics of (Na,K)-ATPase inactivation by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (45, 46). However, the analysis of the single site model presented here emphasizes the fact that the interpretation of the kinetic data in terms of two ATP sites is not unique, so that these data cannot be used to distinguish between one-site and two-site mechanisms.

The new information presented here and in the accompanying paper raises questions on current ideas about the quaternary structure of the functional (Na+, K+) pump. It has been suggested that the native (Na+, K+)-ATPase is an oligomer, namely an αβ2 tetramer, on the basis of cross-linking studies (47-49), ultracentrifugation and gel filtration studies in detergent-solubilized enzyme (50, 51), or electron microscopy of purified membrane-bound enzyme (52). None of the above studies provide information to distinguish whether the smallest functional unit capable of full (Na+, K+)-ATPase activity may be the αβ protomer, or the αβ2 oligomer. The present results show that the inhibition by TNP-ATP and the complex kinetics of activation by ATP can be accounted for by a single nucleotide site. Since our binding studies are consistent with each αβ protomer containing a nucleotide site and an ouabain site (22), these observations suggest that the smallest functional unit capable of full (Na+, K+)-ATPase activity may be the αβ protomer, not the αβ2 tetramer. Whether or not interactions between αβ protomers have functional significance under physiological conditions remains to be demonstrated experimentally.

Appendix

The kinetic model in Fig. 6 was analyzed by the method of King and Altman (53) as described by Segel (54) and Plowman (55) to obtain the steady state equation for the initial rate of P release as a function of ATP concentration. Fig. 6 as drawn represents the closed geometric figure required for application
of the King-Altman (53) method. Plowman (Ref. 55, p. 161) published the solution for a mechanism exactly of this type. The numbered rate constants in Fig. 6 are given the same subscripts as those used by Plowman. If the initial rate of P, production is measured, the concentration of P, and thus the k, reaction is negligible and the initial rate is given by:

$$v = k[E,P]$$  \( \text{(1)} \)

The simplified expression for the rate of phosphate production as a function of ATP concentration is:

$$v = \frac{aS^3 + bS}{S^3 + dS + e}$$  \( \text{(2)} \)

where E, is the total enzyme concentration, S is the ATP concentration, and a, b, c, d, and e are sums of products of the various rate constants shown in Table IA. The double reciprocal form:

$$\frac{E,}{v} = \frac{e}{S^3 + dS + c}$$

will result in a convex upward curve when ae < bd and bd < ad (56). In the limits of zero and infinite substrate concentration, the convex function following equation 3 may be extrapolated as straight lines to obtain an apparent Vmax and Km for each region of the curve. Expressions for these parameters may be obtained analytically from the limits 1 \( S \rightarrow 0 \) and

$$\frac{1}{S} \rightarrow \infty \text{ of Equation 3 and its first derivative:}$$

$$K_m = \frac{be}{bd - ae} \quad V_{\text{max}} = \frac{b^2}{bd - ae}$$  \( \text{(4)} \)

$$K_m = \frac{ad - bc}{ac} \quad V_{\text{max}} = \frac{a}{c}$$  \( \text{(5)} \)

where the subscripts 1 and 2 refer to low and high substrate concentration, respectively. The K, and Vmax relationships are phenomenological coefficients which define the asymptotes of Equation 3. They do not imply that the low and high substrate activation limits may be treated as independent kinetic modes. Although the proper data are not available for a rigorous test of the model, a crude test by a simulation with the published values of the rate constants for those steps that have been measured. Under initial rate conditions, the ADP concentration is negligible, thus all terms containing ADP k, are set equal to zero. Since the reversal of E2-P formation from E1-P has only been observed under unusual conditions (57, 58), k, is also set equal to zero. An additional simplification is made by using the dissociation constants for ATP binding to the high and low affinity forms: K1 = k1/k2 and K2 = k2/k1. This allows expression of the coefficients a, b, c, d, and e of Equation 3 in terms of nine rate constants: k1, k2, k3, k4, k5, k6, k7, k8, and k9, and two ATP dissociation constants: K and K (Table IA). The model was analyzed with respect to TNP-ATP inhibition by including the K, and K, binding steps of the following scheme:

$$\text{TNP-ATP} + E_1 \rightleftharpoons K_1 \text{TNP-ATP} \cdot E_1 \rightleftharpoons K'$$

$$\rightarrow \text{TNP-ATP} \cdot E_2 \rightleftharpoons K_2 \text{TNP-ATP} + E_2$$

To simplify the analysis, the K' step of the above scheme was disregarded. Inhibition by TNP-ATP is assumed to result from TNP-ATP binding to E1 and E2 as a reversible dead-end inhibitor, which introduces the dissociation constants K, and K, in the form of (1 + [I]/K,1) and (1 + [I]/K,2) as multipliers of certain terms in the expressions for d and e, but not a, b, and c (Table IA).

Table IIA lists the values of the constants used to simulate the dependence of (Na,K)-ATPase activity on ATP concentration. In cases where data were not available, additional assumptions were made to assign a range of values, as explained in Table IIA.

Certain features of the shape of curves predicted by Equation 2 should be noted. As discussed by Segel (Ref. 54, p. 658), V versus S plots of Equation 2 cannot exhibit an intermediate plateau in the region of low substrate concentration. With eel electroplax (Na,K)-ATPase, a true plateau region has not been observed, although Glynn and Karlisch have previously reported that such a plateau region exists for erythrocyte (Na,K)-ATPase (4) and have ruled out a mechanism such as that of Fig. 6 on this basis. As pointed out by Tiedel and Kosshland (62), rate equations containing powers of S greater than S are required in order to obtain intermediate plateau regions in V versus S plots. The one-site model shown in Fig. 6 may be modified to give a rate equation with higher powers of S, simply by including more substrate-binding steps. At present, it is not known whether ATP binding to phosphorylated intermediates such as E-P and P is possible or significant under steady state conditions. Also, the E1 → E, transition assumed here probably involves more steps, i.e., Eσ(K) → E,K → E1 (17, 18) that would introduce more ATP binding intermediates in the reaction pathway. Thus, a one-site mechanism cannot be ruled out simply on the basis of an argument concerning inflection points in the V versus S plot.

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