Simultaneous Binding of Phosphate and TNP-ADP to FITC-Modified Na^+,K^+-ATPase†

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ABSTRACT: Double-reciprocal plots of the rate of ATP hydrolysis by Na^+,K^+-ATPase versus ATP concentration are not linear, and may reflect either two distinct binding sites for ATP or a single ATP binding site whose affinity for the nucleotide alternates between high-affinity and low-affinity states. In order to determine whether multiple nucleotides or nucleotide analogs can bind simultaneously to $Na,^+,K^+$-ATPase, the effects of nucleotides on the hydrolysis of *p*-nitrophenyl phosphate and on the dephosphorylation rate of Na^+,K^+-ATPase modified by fluorescein 5′-isothiocyanate (FITC) were measured. FITC blocks the high-affinity binding site for ATP on the Na^+,K^+-ATPase and inhibits ATP hydrolysis at ATP concentrations as high as 8.3 mM. The hydrolysis of *p*-nitrophenyl phosphate and phosphoenzyme formation from inorganic phosphate and Mg^{2+} were not affected by FITC modification. The *p*-nitrophenylphosphatase activity of unmodified Na^+,K^+-ATPase was stimulated by low concentrations of ATP (10–100 μM) and other nucleotides, and was inhibited at higher nucleotide concentrations. In contrast, there was no effect on *p*-nitrophenyl phosphate hydrolysis by FITC-modified Na^+,K^+-ATPase at ATP concentrations less than 100 μM. The hydrolysis of *p*-nitrophenyl phosphate by FITC-modified Na^+,K^+-ATPase was inhibited at ATP concentrations greater than 100 μM. These observations demonstrate that the effects of ATP acting at high-affinity sites are absent in FITC-modified Na^+,K^+-ATPase but the effects of ATP acting at low-affinity sites are still observed. In unmodified Na^+,K^+-ATPase, the rate of dephosphorylation of the phosphoenzyme formed from inorganic phosphate and Mg^{2+} was inhibited by ATP. Although ATP at concentrations up to 1 mM did not affect the rate of dephosphorylation of the FITC-modified Na^+,K^+-ATPase, TNP-ADP inhibited the dephosphorylation of FITC-modified enzyme. The $K_{0.5}$ for the TNP-ADP effect is 35 μM, considerably higher than the measured K_D for TNP-ADP binding to unmodified Na^+,K^+-ATPase (Moczydlowski & Fortes, 1982). These results demonstrate that it is possible to simultaneously bind phosphate, TNP-ADP, and FITC to the Na^+,K^+-ATPase, and may reflect the presence of both high-affinity and low-affinity nucleotide sites on the enzyme.

Sodium- and potassium-activated adenosine-5′-triphosphatase (Na^+,K^+-ATPase,[1] sodium pump) actively transports sodium ions and potassium ions across cell membranes against electrochemical potential gradients for each ion (Skou, 1988; Glynn, 1990). The energy required for this endergonic process is provided by adenosine 5′-triphosphate (ATP), which is hydrolyzed by the pump during the pump cycle. Several different kinetic models have been proposed to describe the coupling of ATP hydrolysis to ion transport. In one model, originally developed by Albers and Post (Albers, 1967; Post et al., 1969, 1972), the Na^+,K^+-ATPase undergoes consecutive conformational changes associated with the hydrolysis of ATP and the binding and release of ions. The ATP concentration dependence of ATP hydrolysis is biphasic, and in an extension of this model, this observation has been explained by a single ATP binding site on each functional molecule that alternates between high-affinity and low-affinity conformations (Moczydlowski & Fortes, 1982). We will refer to this model as the single-site model. A second model, originally suggested by Repke (Repke & Schön, 1973; Repke & Dittrich, 1979; Stein, 1979; Stein et al., 1983), includes two catalytic α subunits that cooperate to maintain the sodium and potassium gradients across the membrane. Each of the catalytic subunits of a diprotomer, $(\alpha\beta)_2$, undergoes the same conformational transitions described in the single-site model, but the individual conformational states are always 180° out-of-phase with respect to each other. As a result, the simultaneous transport of sodium and potassium, as well as the presence of high- and low-affinity ATP binding sites, is expected in this model. In a third model (Plesner, 1987), the cooperativity of the α subunits suggested by Repke occurs only in the presence of both Na^+ and K^+ ions. The partial reactions, such as Na^+-ATPase or K^+-stimulated phosphatase, are catalyzed by a protomeric enzyme.

Although it is difficult to distinguish experimentally between the models of Repke and Plesner, they both differ from the

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[1] Abbreviations: Na^+,K^+-ATPase, sodium- and potassium-activated adenosine-5′-triphosphatase, EC 3.6.1.37; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; CTP, cytosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; TNP-ADP, 2′(3′)-trinitrophenyladenosine 5′-diphosphate; $Co(NH_3)_4ATP$, cobalt tetraammine adenosine 5′-triphosphate; ErITC, erythrosin 5′-isothiocyanate; FITC, fluorescein 5′isothiocyanate; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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single-site model in that they predict the simultaneous existence of at least two ATP sites on each functional enzyme. Several observations that are consistent with either of these multiple-site models have been described, including phosphorylation of the $Co(NH_3)_4ATP$-modified enzyme by [γ-^{32}P]ATP (Scheiner-Bobis et al., 1987), the formation of diprotomeric crystals (Hebert et al., 1985; Ovchinnikov et al., 1985; Skriver et al., 1989), and a target size determined by radiation inactivation that is consistent with an $(\alpha\beta)_2$ structure (Hah et al., 1985; Jensen & Norby, 1988). $Co(NH_3)_4ATP$ is an ATP analog that inactivates the Na^+,K^+-ATPase by phosphorylation of a low affinity ATP site (Scheiner-Bobis et al., 1987, 1989; Buxbaum & Schoner, 1990). These observations and other data (Plesner, L., & Plesner, 1981; Plesner et al., 1981; Plesner, I. W., & Plesner, 1981; Askari & Huang, 1982; Fukushima et al., 1984; Huang & Askari, 1984; Plesner, 1987) indicate that a single-site model may not be sufficient to accurately describe ATP-coupled ion transport catalyzed by Na^+,K^+-ATPase. Single-site models are also used to describe the reaction mechanisms of other E_1/E_2 ATPases which, like Na^+,K^+-ATPase, alternate between an ATP-dependent and ADP-sensitive phosphoenzyme in the E_1 conformational state and an ADP-insensitive but transport ion-sensitive phosphoenzyme intermediate in the E_2 state (Carvalho et al., 1976; de Meis & Vianna, 1979; Wallmark et al., 1980; Ray & Nandi, 1986; Schatzmann et al., 1982; Schatzmann, 1983). Multiple-site models are also consistent with most of the data from these enzymes, and, consequently, the identification of the most accurate model is difficult.

In this paper, the coexistence on Na^+,K^+-ATPase of both high-affinity and low-affinity binding sites for nucleotides is supported by the observation that the ADP analog TNP-ADP inhibits the dephosphorylation rate of the phosphoenzyme formed from FITC-modified enzyme. FITC inactivates Na^+,K^+-ATPase by covalent modification and prevents the high-affinity binding of ATP to the enzyme (Karlish, 1980; Carilli et al., 1982; Farley et al., 1984; Kirley et al., 1984; Tran & Farley, 1986). The fluorescein moiety of FITC is thought to occupy the position of the adenine ring within the high-affinity ATP binding site (Carilli et al., 1982), and the inhibition of the dephosphorylation rate of the FITC-inactivated enzyme by TNP-ADP, therefore, indicates that the the same functional Na,K-ATPase molecule can bind two nucleotides or nucleotide analogs simultaneously.

MATERIALS AND METHODS

Materials

FITC and TNP-ADP were purchased from Molecular Probes (Eugene, OR). $H_3{}^{32}PO_4$ (285 Ci/mg of P) was from ICN (Irvine, CA). All other chemicals and biochemicals were obtained from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), or J. T. Baker (Phillipsburg, NJ) in the highest commercially available degree of purity.

Methods

Purification of Na^+,K^+-ATPase. Na^+,K^+-ATPase was isolated from pig kidney microsomes using a previously described method (Jorgensen, 1974). The specific activity of the enzyme, as determined by a coupled spectrophotometric assay and by the protein determination method of Lowry (1951), was in the range of 18–25.6 units/mg. One unit of Na^+,K^+-ATPase activity is defined as the hydrolysis of 1 μmol of ATP in 1 min under optimal conditions (Scheiner-Bobis & Schoner, 1985).

Modification of Na^+,K^+-ATPase by FITC. A total of 2 mg of Na^+,K^+-ATPase was incubated in 50 mM Tris-HCl, pH 9.2, 100 mM NaCl, and 5 mM Na_2EDTA for 30 min at room temperature in the dark, with or without 50 μM FITC in 0.5% DMSO (Farley & Faller, 1985). The total volume of each sample was 2 mL. The remaining activity of the FITC-treated enzyme was 0–3 % as determined by a coupled spectrophotometric assay (Scheiner-Bobis & Schoner, 1985).

To remove unreacted FITC, 5 mL of 25 mM imidazole/HCl, 1 mM Na_2EDTA, pH 7.5, were added to each sample, which was spun for 30 min at 4°C and 45,000 rpm in a T865.1 rotor (Sorvall Instruments). The pellets were suspended in 5 mL of the imidazole/EDTA buffer and spun down again. This last step was repeated. The pellets were suspended in 2 mL of 25 mM imidazole/HCl, 1 mM Na_2EDTA, pH 7.5.

Effect of Various Nucleotide Di- and Triphosphates on the p-Nitrophenylphosphatase Activity of the FITC-Modified Na^+,K^+-ATPase and of a Control Enzyme. A total of 57 μg of an FITC-modified enzyme or of a control enzyme was incubated at 37 °C in a total volume of 1 mL containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM *p*-nitrophenyl phosphate. At various times, 150 μL of the mixture was transferred to Eppendorf tubes on ice which contained 1 mL of 1 N NaOH. The amount of the released *p*-nitrophenol was calculated from the absorbance of the solution at 405 nm, with an $\epsilon = 18\ 500\ L\cdot mol^{-1}\cdot cm^{-1}$ (Scheiner-Bobis et al., 1989).

The effect of the various nucleotides on the *p*-nitrophenylphosphatase activity was determined by measuring the activity of 9.5 μg of the FITC-inactivated enzyme or of the control enzyme after 15 min at 37°C in the presence of different concentrations of each nucleotide. The other conditions were the same as before.

The effect of ATP on the *p*-nitrophenylphosphatase activity of the control or of the FITC-inactivated enzyme was measured in the presence of 10 mM NaCl and 2 mM KCl by varying the concentration of ATP. The other conditions are the same as before.

Phosphorylation of the FITC-Inactivated and the Control Na^+,K^+-ATPase by [^{32}P]Orthophosphate. To 2 mL of each enzyme suspension, containing 1 mg of protein/mL, were added 0.2 mL of 100 mM $MgCl_2$, 0.4 mL of 100 mM Tris-HCl, pH 7.5, 1.36 mL of H_2O, and 0.04 mL of 50 mM Tris–$^{32}PO_4$. The final concentrations of the protein and ions in the mixture were 0.5 mg/mL protein, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 12.5 mM imidazole hydrochloride, 0.5 mM Na_2EDTA, pH 7.5, and 0.5 mM Tris–$^{32}PO_4$. The samples were incubated at 37 °C for 20 min. The reaction was stopped by transferring the samples to ice.

Dephosphorylation of the FITC-Inactivated and the Control Enzyme in the Presence of EDTA or EDTA + ATP. All the following steps were carried out on ice. To 200 μL of the phosphorylation reaction mixture containing either the control or the FITC-inactivated enzyme was added 200 μL of 40 mM Tris/EDTA, pH 7.5, or 200 μL of 40 mM Tris/EDTA and 2 mM Tris–ATP, pH 7.5. The dephosphorylation reaction, induced upon the chelation and removal of Mg^{2+} from the medium by EDTA, was interrupted at the times indicated in the figures by the addition of 100 μL of 25% trichloroacetic acid containing 5 mM nonradioactive Tris–phosphate. The samples were spun down for 10 min at 12000*g* in a Speed-Vac bench centrifuge. The pellets were suspended in 1 mL of 5% trichloroacetic acid/5 mM Tris–phosphate and centrifuged again under the same conditions, and the suspension and the centrifugation were repeated. Pellets were

analyzed for radioactivity (Cerenkov). Internal standards for the specific radioactivity of Tris–$^{32}PO_4$ were measured in parallel.

Dephosphorylation of FITC-Labeled Na^+,K^+-ATPase, Induced by EDTA in the Presence of TNP-ADP. The dephosphorylation reaction was induced on ice by the addition of Tris–EDTA. To 150 μL of the phosphorylation reaction (0.5 mg of protein/mL) was added 150 μL of 40 mM Tris–EDTA, pH 7.5, and the reaction was stopped after 11 s by the addition of 75 μL of 25% trichloroacetic acid/5 mM Tris–phosphate. In order to study the TNP-ADP effect on the dephosphorylation rate, the nucleotide was added to the phosphorylation mixture 30 s prior to the addition of the EDTA solution. Although TNP-ADP reduces the dephosphorylation rate even when applied simultaneously with the EDTA solution, the largest effect can be observed when the ATP analog is added to the enzyme solution prior to the addition of EDTA. For the 0-s value, the enzyme was precipitated by trichloroacetic acid prior to the addition of the EDTA solution.

After the addition of trichloroacetic acid, the samples were spun for 8 min at 12000*g* in the Speed-Vac centrifuge. The supernatant was removed by aspiration. The pellets were washed twice with 1 mL of 5% trichloroacetic acid/5 mM Tris–phosphate, each time followed by a centrifugation, and were counted for radioactivity (Cerenkov). An internal radioactivity standard for the specific radioactivity of Tris–$^{32}PO_4$ was measured in parallel.

Effect of Ions and Ouabain on the Dephosphorylation of an FITC-Modified Enzyme in the Presence of TNP-ADP. The conditions for the phosphorylation and the dephosphorylation of the enzyme were the same as described before. The effect of Na^+, K^+, and ouabain was studied as follows: To 100 μL of the phosphorylation reaction containing 25 μg of phosphorylated FITC–enzyme was added 2 μL of 10 mM TNP-ADP. After 30 s on ice, 98 μL of a solution containing EDTA and either KCl, NaCl, KCl + ouabain, or NaCl + ouabain was added to the sample. After the two solutions were mixed, the final concentrations were 100 μM TNP-ADP, 20 mM Tris–EDTA, pH 7.5, 20 mM KCl, 20 mM NaCl, and 100 μM ouabain. The dephosphorylation was interrupted at various times by the addition of 50 μL of 25% trichloroacetic acid/5 mM Tris–phosphate. The samples were washed with 5% trichloroacetic acid/5 mM Tris–phosphate, and were measured for radioactivity as described above. The effects of K^+ or Na^+ were also studied in the absence of TNP-ADP.

RESULTS

Properties of the p-Nitrophenylphosphatase Activity of the FITC-Inactivated Na^+,K^+-ATPase. Incubation of Na^+,K^+-ATPase with approximately stoichiometric concentrations of FITC leads to complete inactivation of ATP hydrolysis (Karlish, 1980; Carilli et al., 1982) as a result of covalent modification of K501 on the α subunit of the enzyme (Farley et al., 1984; Kirley et al., 1984). The amino acid sequence ^{501}KGAPER around the modified lysine is conserved in most P-type ion transport ATPases (Michinson et al., 1982; Farley et al., 1984; Farley & Faller, 1985; MacLennan et al., 1985; Shull et al., 1985; Shull & Lingrel, 1986; Brandl et al., 1986). ATP protects the enzyme against inactivation by FITC, and FITC modification blocks the high-affinity binding of ATP to Na^+,K^+-ATPase (Tran & Farley, 1986). These observations have led to the suggestion that FITC inhibits Na^+,K^+-ATPase and other ATPases by covalent modification of the high-affinity ATP binding site (Pick & Karlish, 1980; Karlish, 1980; Carilli et al., 1982; Michinson et al., 1982; Farley et al., 1984; Farley & Faller, 1985).

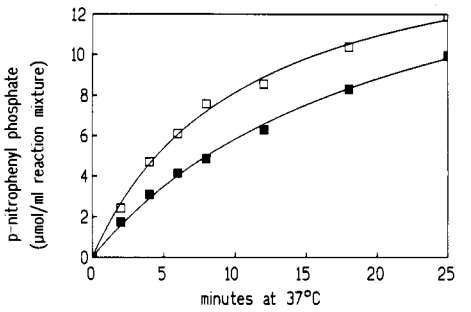

FIGURE 1: Phosphatase activity of a control and a FITC inactivated Na^+,K^+-ATPase. A total of 57 μg of a control (□) or an FITC-modified Na^+,K^+-ATPase (■) was incubated with 5 mM KCl, 5 mM $MgCl_2$, and 5 mM *p*-nitrophenyl phosphate in 10 mM Tris–HCl, pH 7.5. The phosphatase activity was determined at the times indicated on the abscissa from the absorbance of *p*-nitrophenol at 405 nm.

Inhibition of the pump cycle of the Na^+,K^+-ATPase by FITC does not affect all of the partial reactions of the pump. While activities of the enzyme associated with a high affinity for ATP, such as ATP binding (K_D = 0.1–0.2 μM) or phosphoenzyme formation from ATP or Na^+-ATPase, are inhibited by FITC, the activities of the pump that are associated with a low affinity for ATP are still intact (Karlish, 1980). Thus, the stimulation of *p*-nitrophenyl phosphate hydrolysis by K^+, the inhibition of *p*-nitrophenyl phosphate hydrolysis by high concentrations of ATP, and the formation of phosphoenzyme from inorganic phosphate are unaffected by the modification. In Figure 1, the *p*-nitrophenylphosphatase activity of the FITC-modified enzyme is compared to the phosphatase activity of unmodified Na^+,K^+-ATPase. Although ATP hydrolysis by the FITC-modified enzyme was only 2% of the control enzyme in this experiment, hydrolysis of *p*-nitrophenyl phosphate by the FITC-modified enzyme occurred at nearly the same rate as hydrolysis by the control enzyme. Other phosphoesters and phosphoanhydrides can also be hydrolyzed by FITC-modified Na^+,K^+-ATPase (Davis & Robinson, 1988). Additional experiments in this laboratory have verified that the FITC-modified enzyme can bind [^{32}P]phosphate, phosphate, and [^{3}H]ouabain, and can also occlude $^{86}Rb^+$ ions to the same extent as the nonmodified enzyme (data not shown).

The *p*-nitrophenylphosphatase activity can be inhibited to almost 100% in both the FITC-modified enzyme and the control enzyme by the addition of ADP or ATP. The K_i values for inhibition of the *p*-nitrophenylphosphatase reaction were obtained from Dixon plots (data not shown), and are summarized in Table I. Both ADP and ATP inhibit the FITC-labeled enzyme with very similar inhibition constants, which are similar to the K_i for the inhibition of the *p*-nitrophenylphosphatase reaction of the control enzyme by ATP.

The effects of ITP, GTP, CTP, AMP, and PO_4(Tris) on the phosphatase reaction of the FITC-modified enzyme are also summarized in Table 1. Although all of the nucleotides inhibit the phosphatase reaction, the adenine nucleotides inhibit with the lowest K_i values, consistent with the hypothesis that the site where the nucleotides bind retains the capacity to distinguish between different purines and pyrimidines. The K_i for phosphate inhibition is 1.5 mM, similar to the inhibition constants for ATP or ADP (Table I).

In contrast to the inhibition of *p*-nitrophenyl phosphate hydrolysis by high concentrations of ATP, low concentrations of the nucleotide, in the presence of low concentrations of NaCl and KCl, stimulate the *p*-nitrophenylphosphatase

Table I: Inhibition of the *p*-Nitrophenylphosphatase Activity of FITC-Modified and Unmodified Na^+,K^+-ATPase[a]

inhibitor used	K_i (mM)
FITC-Modified Na,K-ATPase	
phosphate	1.52
AMP	17.50
ADP	1.75
ATP	1.50
GTP	4.25
CTP	6.72
ITP	7.75
Unmodified Na,K-ATPase	
phosphate	1.90
AMP	10.50
ATP	1.67

[a] The *p*-nitrophenylphosphatase activity of FITC-modified or control enzyme was determined in the presence of various concentrations of nucleotides or phosphate as described under *Methods*. The K_i values were determined from Dixon plots. *Inhibitor used* refers to phosphate or the nucleotide used to inhibit the hydrolysis of *p*-nitrophenyl phosphate. The K_i values were determined from a single experiment.

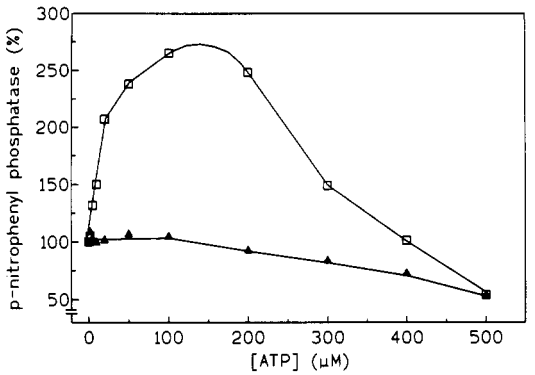

FIGURE 2: Effects of low concentrations of ATP on the hydrolysis of *p*-nitrophenyl phosphate by control and FITC-inactivated Na^+,K^+-ATPase.The effect of various concentrations of ATP on the *p*-nitrophenylphosphatase reaction of unmodified (□) or of FITC-modified (▲) Na^+,K^+-ATPase was measured as described in Figure 1 in the presence of 10 mM NaCl and 2 mM KCl.

activity of native Na^+,K^+-ATPase (Robinson, 1970; Pitts & Askari, 1970). Figure 2 shows that the stimulation of *p*-nitrophenyl phosphate hydrolysis that is observed with unmodified Na^+,K^+-ATPase in the presence of low concentrations of ATP and 10 mM NaCl and 2 mM KCl is absent in FITC-modified enzyme. Inhibition of enzymatic activity by concentrations of ATP greater than 100 μM, however, is still observed.

ATP Effect on the Dephosphorylation Rate. Na^+,K^+-ATPase that has been phosphorylated by inorganic phosphate in the presence of Mg^{2+} is rapidly dephosphorylated when EDTA is added (Askari & Huang, 1982). The rate of dephosphorylation can be considerably reduced, however, when ATP is added simultaneously with EDTA (Askari & Huang, 1982). Figure 3 shows the effects of 1 mM ATP on the dephosphorylation of a control and of an FITC-modified enzyme. The phosphorylation capacity for the control and the FITC enzyme was the same in these experiments (control enzyme = 4.7 nmol of phosphate/mg; FITC enzyme = 4.2 nmol of phosphate/mg). As reported previously (Askari & Huang, 1982), ATP can bind to the unmodified enzyme in the E_2–P conformation and reduce the dephosphorylation rate. Since ATP does not affect the dephosphorylation rate of the FITC-inactivated enzyme, either the site where ATP binds is inaccessible to ATP when FITC is covalently bound to the enzyme or the affinity of the enzyme for ATP is markedly reduced by FITC modification.

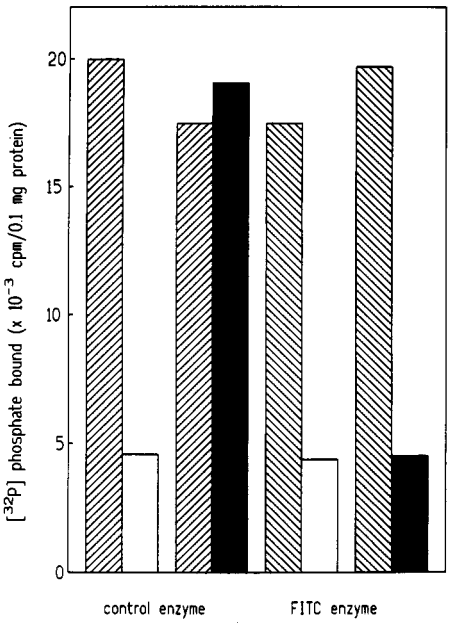

FIGURE 3: Dephosphorylation of a control and a FITC inactivated Na^+,K^+-ATPase.A total of 100 μg of a control Na^+,K^+-ATPase or the same amount of FITC-modified enzyme (remaining activity 0.3%) was phosphorylated by 0.5 mM Tris–$^{32}PO_4$ in the presence of 5 mM $MgCl_2$. In three repetitions of this experiment, the average phosphorylation capacity was 4.7 nmol of phosphate/mg for the control enzyme and 4.2 nmol of phosphate/mg for the FITC-modified enzyme. In all lanes the hatched bars show the level of phosphorylation for the different enzymes before initiation of dephosphorylation by 20 mM Tris–EDTA. The white bars show the level of phosphoenzyme observed after the addition of Tris–EDTA. The black bars show the level of phosphoenzyme after addition of Tris-EDTA + 1 mM ATP.

Effect of TNP-ADP on the Dephosphorylation Rate of the FITC-Modified Enzyme. TNP-ATP, like ATP, inhibits the phosphatase reaction of the FITC-modified Na^+,K^+-ATPase (Davis & Robinson, 1988). TNP-ATP and TNP-ADP bind to the ATP binding site of the Na^+,K^+-ATPase with a K_D between 40 and 700 nM, depending upon temperature (Moczydlowski & Fortes, 1981). This is the highest known affinity for a substrate or an ATP analog of the Na^+,K^+-ATPase. Like ATP, TNP-ADP inhibits the dephosphorylation of Na^+,K^+-ATPase (data not shown), and in order to distinguish on FITC-modified Na^+,K^+-ATPase between an inaccessible nucleotide binding site and a binding site with reduced affinity for nucleotides, the effects of TNP-ADP on the dephosphorylation rate of the FITC-modified Na^+,K^+-ATPase were examined. Figure 4 demonstrates that TNP-ADP, when added simultaneously with EDTA, slows down the dephosphorylation rate of the FITC-inactivated enzyme. The $K_{0.5}$ value for this effect is 35 μM, indicating that the FITC-modified Na^+,K^+-ATPase has an apparent affinity for TNP-ADP that is much lower than the affinity of the unmodified enzyme for this nucleotide (Moczydlowski & Fortes, 1981). This suggests that the site at which ATP binds to inhibit dephosphorylation of the phosphoenzyme is present on the FITC-modified enzyme but that its affinity for ATP is reduced by the modification.

In order to test whether the catalytic ATP binding site is also retained in FITC-modified enzyme, but with reduced affinity for ATP, the hydrolysis of ATP was measured at high ATP concentrations. No hydrolysis of ATP by FITC-modified enzyme was detected in either Na^+,K^+-ATPase or Na^+-ATPase reactions at concentrations of ATP up to 8.3 mM (data not shown). Higher concentrations of ATP inhibited the activity of the unmodified enzyme.

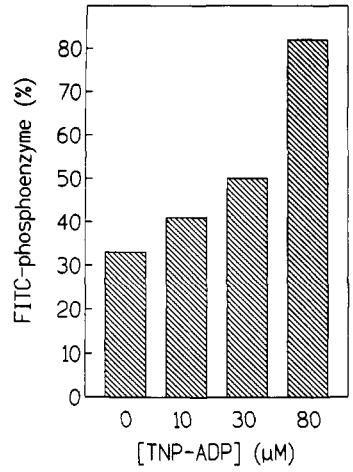

FIGURE 4: TNP-ADP-induced inhibition of the dephosphorylation of FITC modified Na^+,K^+-ATPase. A total of 75 μg of FITC-modified and Tris-$^{32}PO_4$-phosphorylated Na^+,K^+-ATPase was preincubated for 30 s with the various concentrations of TNP-ADP indicated on the abscissa. The dephosphorylation of the FITC-modified enzyme was induced by 20 mM Tris-EDTA. After 11 s on ice, the dephosphorylation reaction was interrupted by 5% trichloroacetic acid/5 mM PO_4.

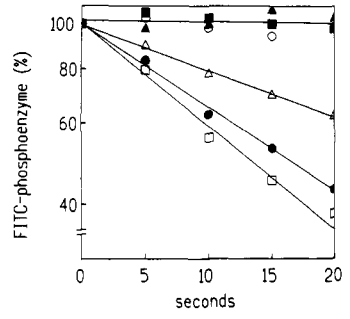

FIGURE 5: Effect of Na^+, K^+ and ouabain on the TNP-ADP induced inhibition of the dephosphorylation of the FITC inactivated Na^+,K^+-ATPase. The conditions for the phosphorylation were the same as described in Figure 4. The dephosphorylation was induced by 20 mM Tris–EDTA, pH 7.5, after preincubating 25 μg of FITC-modified Na^+,K^+-ATPase with 100 μM TNP-ADP. The different symbols in the figure represent the following conditions: (□) FITC–enzyme + 20 mM Tris-EDTA; (■) FITC–enzyme + 20 mM Tris–EDTA + 100 μM TNP-ADP; (●) FITC–enzyme + 20 mM Tris–EDTA + 100 μM TNP-ADP + 20 mM KCl; (○) FITC–enzyme + 20 mM Tris–EDTA + 100 μM TNP-ADP + 20 mM KCl + 100 μM ouabain; (△) FITC–enzyme + 20 mM Tris–EDTA + 100 μM TNP-ADP + 20 mM NaCl; (▲) FITC–enzyme + 20 mM Tris–EDTA + 100 μM TNP-ADP + 20 mM NaCl + 100 μM ouabain. The 100% value in this figure equals to 3.85 (± 0.15) nmol of phosphate/mg of protein.

Effect of Ions and Ouabain on the TNP-ATP-Induced Reduction of the Dephosphorylation Rate. Previous studies have shown that the inhibition of dephosphorylation by ATP is reversed by Na^+, but not by K^+ (Askari & Huang, 1982). In the experiments summarized in Figure 5, TNP-ADP decreased the dephosphorylation rate of FITC-modified enzyme, and this effect of TNP-ADP was prevented by the presence of 20 mM Na^+. In contrast to the absence of an effect of K^+ on the dephosphorylation rate of the native Na^+,K^+-ATPase in the presence of ATP, K^+ also accelerates the dephosphorylation of FITC-inactivated enzyme in the presence of TNP-ADP. In the absence of TNP-ADP and EDTA, but under otherwise analogous conditions, K^+ does not accelerate the dephosphorylation rate of either the control or the FITC-inactivated enzyme (data not shown). In the presence of TNP-ADP, ouabain blocks the Na^+- or K^+-induced acceleration of the dephosphorylation of the FITC-inactivated enzyme, most likely by inducing a more stable FITC–phosphoenzyme (Figure 5).

DISCUSSION

The experiments described in this report were undertaken to determine whether two nucleotides or nucleotide analogs could bind simultaneously to each functional Na^+,K^+-ATPase molecule. Since the simultaneous presence of multiple nucleotide binding sites is not accommodated by single-site kinetic models of Na^+,K^+-ATPase, identification of two ATP binding sites would suggest that more complex models may be required to describe the mechanism of ATP-coupled ion transport.

The K^+-stimulated *p*-nitrophenylphosphatase activity of Na^+,K^+-ATPase previously modified with FITC was investigated. As has been previously observed, the FITC-modified enzyme hydrolyzes *p*-nitrophenyl phosphate at a rate comparable to that of control Na^+,K^+-ATPase (Figure 1). Low concentrations of ATP stimulate this reaction in unmodified Na^+,K^+-ATPase, and concentrations of ATP above 100 μM inhibit the reaction (Figure 2). In contrast, with FITC-modified enzyme, there was no stimulation of *p*-nitrophenyl phosphate hydrolysis by low concentrations of ATP, although inhibition by ATP concentrations greater than 100 μM was still observed. These data suggest that the effects of ATP on *p*-nitrophenyl phosphate hydrolysis by Na^+,K^+-ATPase occur at two distinct sites and that FITC modification blocks a high-affinity ATP binding site, but that a low-affinity site is still present.

ATP reduces the dephosphorylation rate of Na^+,K^+-ATPase that has been phosphorylated by inorganic phosphate when the dephosphorylation is induced by chelation of Mg^{2+} by EDTA (Askari & Huang, 1982). The $K_{0.5}$ for this effect was about 0.2 mM. This observation was confirmed and was compared to the effect of ATP on the dephosphorylation of phosphoenzyme generated similarly but from FITC-modified enzyme. As shown in Figure 3, 1 mM ATP had no effect on the dephosphorylation rate of the FITC-modified enzyme. When the experiments were repeated using TNP-ADP instead of ATP (Figure 4), however, the EDTA-induced dephosphorylation rate of the FITC-modified enzyme was decreased significantly. The $K_{0.5}$ value for the effect of TNP-ADP on dephosphorylation is 35 μM. Assuming that this represents the apparent affinity of the FITC–enzyme for the nucleotide, the affinity for TNP-ADP is reduced at least 100-fold when compared to the affinity of the unmodified enzyme at the same temperature (K_D = 0.2–0.5 μM; Moczydlowski & Fortes, 1981). If the affinity of the FITC-modified enzyme for ATP is reduced similarly, the $K_{0.5}$ for an effect of ATP on the dephosphorylation rate would be at least 20 mM, and the absence of a measurable effect of 1 mM ATP on the dephosphorylation of FITC-modified Na^+,K^+-ATPase is not surprising. No hydrolysis of ATP by FITC-modified Na^+,K^+-ATPase was detected at concentrations of ATP up to 8.3 mM. Thus, the modification of Na^+,K^+-ATPase by FITC appears to block the catalytic ATP site, and to reduce the affinity of the enzyme for the nucleotide at a low-affinity site, but without rendering that site inaccessible.

The specificity of the binding of TNP-ADP to the FITC-modified Na^+,K^+-ATPase was investigated by measuring the effects of substrate ions and ouabain on the dephosphorylation rate. It has previously been shown that Na^+ ions can overcome the effect of ATP on dephosphorylation but that K^+ ions have no effect on the dephosphorylation rate (Askari & Huang, 1982). Consistent with these results, Na^+ induces an accel-

erated dephosphorylation of the phosphoenzyme that had previously been stabilized by TNP-ADP (Figure 5). In contrast, however, K^+ also accelerates the decay of the FITC–phosphoenzyme. Although the reasons for this difference are not clear, the observation suggests that the conformations of the phosphoenzyme obtained from Mg^{2+} and phosphate are not identical in unmodified and FITC-modified Na^+,K^+-ATPase. As discussed by others (Post et al., 1975; Askari & Huang, 1982), multiple forms of the phosphoenzymes may be obtained from ATP or phosphate under various conditions. The absence of an effect of K^+ on the dephosphorylation of phosphoenzyme was interpreted by Huang and Askari (1982) to indicate that this phosphoenzyme was different from E_2P. The sensitivity to K^+ of dephosphorylation of the phosphoenzyme formed from FITC-modified Na^+,K^+-ATPase may indicate that the enzyme is in the E_2P conformation; however, this is not a unique interpretation. K^+ has been shown to reduce the affinity of Na^+,K^+-ATPase for ATP analogs at a low-affinity site (Scheiner-Bobis et al., 1987). Ouabain prevents the reversal of the TNP-ADP effect by either Na^+ or K^+. Ouabain is a cardioactive steroid which specifically binds to the phosphorylated Na^+,K^+-ATPase and inhibits the enzyme by formation of a stable phosphoenzyme–ouabain complex (Wallick & Schwartz, 1988). The prevention of the dephosphorylation is probably due to the formation of a stable FITC–phosphoenzyme-ouabain complex.

The results described above indicate that FITC, TNP-ADP, and phosphate can be bound simultaneously to Na^+,K^+-ATPase. The locations of the binding sites on the enzyme for each ligand are not known. If FITC occupies the high-affinity ATP binding site (Karlish, 1980; Carilli et al., 1982; Farley et al., 1984; Kirley et al., 1984), the results are consistent with the existence of two nucleotide binding sites on the enzyme. The evidence that FITC occupies the high-affinity ATP binding site on Na^+,K^+-ATPase is kinetic and, therefore, may have other interpretations. The failure of ATP to bind with high affinity to FITC-modified Na^+,K^+-ATPase (Tran & Farley, 1986), for example, could occur if the FITC modification locks the Na^+,K^+-ATPase in the E_2 conformation. In the E_2 conformation, the properties of the high-affinity ATP site would be absent but the properties of the low-affinity site would remain. Although this interpretation is supported by the observation that trypsin digestion of FITC-modified Na^+,K^+-ATPase in the presence of either NaCl or KCl generates only peptide fragments identified with the E_2 conformation (Carilli et al., 1982), it is not consistent with the observed changes in fluorescein fluorescence intensity that occur when FITC-modified Na^+,K^+-ATPase is alternately exposed to solutions containing Na^+ and K^+ ions (Karlish, 1980; Faller et al., 1991; Abbott et al., 1991). These experiments indicate that the FITC-modified enzyme can exist in either E_1 or E_2 conformations and that transitions between the two conformations, with rate constants comparable to those observed with unmodified enzyme, can be induced by monovalent cations.

The effects of TNP-ADP on dephosphorylation of FITC-modified Na^+,K^+-ATPase could also be understood if the FITC modification did not block the high-affinity ATP binding site but simply reduced the affinity of the enzyme for ATP by reaction with K501 outside of the nucleotide binding site. This is the explanation suggested by Champeil et al. (1988) for the effects of high concentrations of ATP on the dephosphorylation rate of FITC-modified Ca^{2+}-ATPase. Although TNP-ADP has the same inhibitory effect on the dephosphorylation rate of the FITC-modified Na^+,K^+-ATPase as ATP or ADP have on the dephosphorylation rate of the native enzyme (Askari & Huang, 1982), ATP binds to the phosphorylated Ca^{2+}-ATPase from sarcoplasmic reticulum and increases the dephosphorylation rate of this enzyme (Champeil et al., 1988). The rate constant for dephosphorylation of unmodified Ca^{2+}-ATPase was increased 2-fold by 10–100 μM Tris–ATP or Na–ATP, but in this concentration range, neither salt of ATP had any effect on the dephosphorylation of FITC-modified enzyme. The rate of dephosphorylation of FITC-modified enzyme was increased 2-fold, however, by 1–10 mM Tris–ATP, and it was suggested that the effect of FITC modification is a reduction in the affinity of the enzyme for ATP by modification near, but not within, the ATP binding site. The reasons for the different effects of ATP on the dephosphorylation rates of unmodified Na^+,K^+-ATPase and Ca^{2+}-ATPase are not known, however, and as suggested above, a reduction in the affinity of the FITC-modified enzymes for the nucleotide that affects the dephosphorylation rate does not exclude modification within a high-affinity catalytic binding site. Although it is difficult to exclude the possibility that FITC reacts outside of the high-affinity ATP binding site without knowing the structure of the protein, we have recently identified G502 as the amino acid modified in Na^+,K^+-ATPase by 2-N_3-ATP.[2] Since 2-N_3-ATP is a substrate for the Na^+,K^+-ATPase, the likelihood that the FITC binding site and the high-affinity ATP binding site overlap at least partially seems high. This interpretation is also supported by the observation that an iodinated fluorescein derivative binds to yeast hexokinase (Fletterich et al., 1975) and lactate dehydrogenase (Wassarman & Lentz, 1971) at the site normally occupied by the adenine ring of ATP.

The largest increase in the dephosphorylation rate of the Ca^{+2}-ATPase was also attributed to Na^+ ions at concentrations greater than 1 mM, and it is possible that the effects observed at high ATP concentrations were caused by high ionic strength or Tris, which is known to mimic the effects of sodium on the Na^+,K^+-ATPase (Skou & Esmann, 1980). The effects of TNP-ATP or TNP-ADP on the dephosphorylation rate of the phosphorylated Ca^{2+}-ATPase have not been described.

Reaction with FITC does appear to reduce the affinity of the enzyme for ATP, but at the low-affinity site rather than at the high-affinity catalytic site, as suggested by the $K_{0.5}$ of FITC-modified Na^+,K^+-ATPase for TNP-ADP. If the effect of FITC modification were to reduce the affinity of the enzymes for substrate ATP by a factor of about 100 (Champeil et al., 1988), some hydrolysis of ATP by FITC-modified Na^+,K^+-ATPase might be expected at high ATP concentrations. No hydrolysis of ATP at concentrations up to 8.3 mM, however, was detected. Although this concentration of ATP would not saturate a site with a K_M of 20–30 mM, detection of substrate hydrolysis at the calculated fractional occupancy of such a site is within the limits of the assay used; 5 mM ATP has also been observed to have no effect on the fluorescence changes induced in FITC-modified Na^+,K^+-ATPase by Na^+ or K^+ ions (Karlish, 1980). If reaction outside of a single ATP binding site is responsible for the effects of FITC modification on Na^+,K^+-ATPase that are described in this report, a much larger reduction in the binding affinity for ATP than was inferred for Ca^{2+}-ATPase must occur. Nevertheless, this possibility cannot be completely excluded without a high-resolution structure.

Kinetic effects of high concentrations of ATP or of TNP-ATP on the hydrolysis of 3-*O*-methylfluorescein phosphate

[2] C. M. Tran, E. E. Huston, and R. A. Farley, manuscript in preparation.

by FITC-modified Na^+,K^+-ATPase were suggested to occur by displacement of FITC out of the catalytic site by the nucleotides (Davis & Robinson, 1988). The fluorescein moiety remains tethered to K501 but no longer blocks access to the nucleotide binding site. More recent data, however, suggest that FITC does not significantly reorient during conformational changes associated with substrate hydrolysis. The fluorescence polarization anisotropy (r) of FITC-modified Na^+,K^+-ATPase is 0.336, close to the value of $r_0 = 0.390$ expected in the absence of rotational motion of the fluorescein moiety, and neither the anisotropy nor the motional restriction of fluorescein is affected during the $E_1Na \leftrightarrow E_2K$ conformational change (Abbott et al., 1991). Thus, it seems unlikely that either ATP or 3-*O*-methylfluorescein phosphate displaces FITC from the catalytic site on the enzyme. In additional experiments, Amler et al. (1992) showed that the modification of Na^+,K^+-ATPase at the ATP binding site by FITC is competitively inhibited by ErITC. After enzyme activity had been inhibited approximately 50% by FITC, presumably by modification of 50% of the nucleotide sites, the enzyme was modified by ErITC, and the distance between the two fluorescent probes, representing the distance between ATP sites, was estimated to be between 3.3 and 6.6 nm. A similar distance was estimated for the FITC and TNP-ADP probes, indicating that the binding sites for these two molecules on Na^+,K^+-ATPase are quite far apart. An oligomeric $(\alpha\beta)_2$ structure was considered to be the most likely explanation for these observations, however, energy transfer between noninteracting $\alpha\beta$ protomers that maintain a fixed orientation with respect to each other in the plane of the membrane was not excluded.

The results described in this report provide additional support for the suggestion that multiple ATP binding sites exist on each functional Na^+,K^+-ATPase molecule. Whether the high-affinity and low-affinity sites reside on the same α subunit or on different α subunits in a diprotomeric complex, however, is not known. The results do not exclude a partial overlap of the two sites, possibly induced by a conformational change as the enzyme moves through the catalytic cycle. In this case, the high-affinity ATP binding site could expand in size as the enzyme undergoes a conformational change, thereby reducing the apparent affinity for ATP and also allowing a second nucleotide to bind. A reaction intermediate that simultaneously binds two ATP molecules, however, does not appear in single-site kinetic models of Na^+,K^+-ATPase, in which a single ATP site alternates between high-affinity and low-affinity states (Moczydlowski & Fortes, 1981; Smith et al., 1980). The results described in this report, therefore, support earlier suggestions that extensions of these models which include multiple nucleotide binding sites should be considered.

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REFERENCES

Abbott, A. J., AmLer, E., & Ball, W. J., Jr., (1991) *Biochemistry 30*, 1692–1701.
Albers,R. W. (1967) *Annu. Rev. Biochem. 36*, 727–756.
Amler, E., Abbott, A., & Ball, W. J., Jr. (1992) *Biophys. J. 61*, 553–568.
Askari, A., & Huang, W.-H. (1982) *Biochem. Biophys. Res. Commun. 104*, 1447–1453.
Brandl, C. J., Green, N. M., Korczak, B., & MacLennan, D. H. (1986) *Cell 44*, 597–607.
Buxbaum, E., & Schoner, W. (1990) *Eur. J. Biochem. 195*, 407–419.
Carilli, C. T., Farley, R. A., Perlman, D. M., & Cantley, L. C. (1982) *J. Biol. Chem. 257*, 5601–5606.
Carvalho,M., de Souza, D., & de Meis, L. (1976) *J. Biol. Chem. 251*, 3629–3636.
Champeil, P., Riollet, S., Orlowski, S., Guillain, F., Seebregts, C. J., & McIntosh, D. B. (1988) *J. Biol. Chem. 263*, 12288–12294.
Davis, R. L., & Robinson, J. D. (1988) *Biochim. Biophys. Acta 953*, 26–36.
de Meis, L., & Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275–292.
Faller, L. D., Diaz, R. A., Scheiner-Bobis, G., & Farley, R. A. (1991) *Biochemistry 30*, 3503–3510.
Farley, R. A., & Faller, L. D. (1985) *J. Biol. Chem. 260*, 3899–3901.
Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535.
Fletterick, R. J., Bates, D. J., & Steitz, T. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 38–42.
Fukushima, Y., Yamada, S., & Nakao, M. (1984) *J. Biochem. (Tokyo) 95*, 356–369.
Glynn, I. M. (1990) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) 2nd ed., pp 35–114.
Hah, J., Goldinger, J. M., & Jung, C. Y. (1985) *J. Biol. Chem. 260*, 14016–14019.
Hegyvary, C., & Post, R. L. (1971) *J. Biol. Chem. 246*, 5234–5240.
Huang, H.-W., & Askari, A. (1984) *J. Biol. Chem. 259*, 13287–13291.
Jensen, J., & Norby, J. G. (1988) *J. Biol. Chem. 263*, 18063–18070.
Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.
Kirley, T. L., Wallick, E. T., & Lane, L. K. (1984) *Biochem. Biophys. Res. Commun. 125*, 767–773.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacLennan, D. H., Brandl, C. J., Korczak, B., & Green, N. M. (1985) *Nature 316*, 696–700.
Mitchinson, C., Wilderspin, A. F., Trinaman, B. J., & Green, N. M. (1982) *FEBS Lett. 146*, 87–92.
Moczydlowski, E. G., & Fortes, P. A. G. (1981) *J. Biol. Chem. 256*, 2346–2356.
Nagamune, H., Urayama, O., Hara, Y., & Nakao, M. (1986) *J. Biochem. 99*, 1613–1624.
Ovchinikov, Y. A., Demin, V. V., Barnakov, A. N., Kuzin, A. P., Lunev, A. V., Modyanov, N. N., & Dzhandzhugazyan, K. N. (1985) *FEBS Lett. 190*, 73–76.
Pick, U., & Basilian, S. (1981) *FEBS Lett. 123*, 127–130.
Pitts, B. J. R., & Askari, A. (1971) *Biochim. Biophys. Acta 227*, 453–459.
Plesner, I. W. (1987) *Biophys. J. 51*, 69–78.
Plesner, L., & Plesner, I. W. (1981) *Biochim. Biophys. Acta 643*, 449–462.
Plesner, I. W., & Plesner, L. (1981) *Biochim. Biophys. Acta 648*, 231–246.
Plesner, I. W., Plesner, L., Norby, J. G., & Klodos, I. (1981) *Biochim. Biophys. Acta 643*, 483–494.
Post, R. L., Kume, L., Tobin, T., Orcutt, H. B., & Sen, A. K. (1969) *J. Gen. Physiol. 54*, 306–326.
Post, R. L., Hegyvary, C., & Kume, S. (1972) *J. Biol. Chem. 247*, 6530–6540.
Post, R. L., Toda, G., & Rogers, F. N. (1975) *J. Biol. Chem. 250*, 691–701.
Ray, T. K., & Nandi, J. (1986) *Biochem. J. 233*, 231–238.
Repke, K. R. H., & Schön, R. (1973) *Acta Biol. Med. Ger. 31*, K19-K30.

Repke, K. R. H., & Dittrich, F. (1979) in *Na+,K+-ATPase, Structure and Kinetics* (Skou, J. C., & Norby, J. B., Eds.) pp 487–502, Academic Press, London, New York, and San Francisco.

Robinson, J. D. (1970) *Arch. Biochem. Biophys. 139*, 164–171.

Robinson, J. D., Leach, C. A., Davis, R. L., & Robinson, L. J. (1986) *Biochim. Biophys. Acta 872*, 294–304.

Schatzmann, H. J. (1983) *Annu. Rev. Physiol. 45*, 303–312.

Schatzmann, H. J., Buergin, H., Luterbacher, S., Stieger, J., Wuethrich, A., & Rossi, J. P. (1982) *Horm. Cell Regul. 6*, 13–25.

Scheiner-Bobis, G., & Schoner, W. (1985) *Eur. J. Biochem. 152*, 739–746.

Scheiner-Bobis, G., Fahlbusch, K., & Schoner, W. (1987) *Eur. J. Biochem. 168*, 123–131.

Scheiner-Bobis, G., Esmann, M., & Schoner, W. (1989) *Eur. J. Biochem. 183*, 173–178.

Schoner, W., Beusch, R., & Kramer, R. (1968) *Eur. J. Biochem. 7*, 102–110.

Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., Zhao, R. S., & de Pont, J. J. H. H. M (1986) *Biochim. Biophys. Acta 861*, 259–266.

Shull, G. E., & Lingrell, J. B. (1986) *J. Biol. Chem. 261*, 16788–16791.

Shull, G. E., Schwartz, A., & Lingrell, J. B. (1985) *Nature 316*, 691–695.

Skou, J. C. (1988) *Methods Enzymol. 156*, 1–25.

Skou, J. C., & Esmann, M. (1980) *Biochim. Biophys. Acta 601*, 386–402.

Skriver, E., Maunsbach, A. B., Hebert, H., Scheiner-Bobis, G., & Schoner, W. (1989) *J. Ultrastruct. Mol. Struct. Res. 102*, 189–195.

Smith, R. L., Zinn, K., & Cantley, L. C. (1980) *J. Biol. Chem. 255*, 9852–9859.

Stein, W. D. (1979) in *Na+,K+-ATPase, Structure and Kinetics* (Skou, J. C., & Norby, J. B., Eds.) pp 475–486, Academic Press, London, New York, and San Francisco.

Stein, W. D., Lieb, W. R., Karlish, S. J. D., & Eilam, Y (1983) *Proc. Natl. Acad. Sci. U.S.A. 70*, 275–278.

Tran, C. M., & Farley, R. A. (1986) *Biochim. Biophys. Acta 860*, 9–14.

Wallick, E. T., & Schwartz, A. (1988) *Methods Enzymol. 156*, 201–229.

Wallmark, B., Stewart, H. B., Rabon, E., Saccomani, G., & Sachs, G. (1980) *J. Biol. Chem. 255*, 5313–5319.

Wassarman, P. M., & Lentz, P. J., Jr. (1971) *J. Mol. Biol. 60*, 509–522.