Nucleophilic Behavior of Lysine-501 of the α-Polypeptide of Sodium and Potassium Ion Activated Adenosinetriphosphatase Consistent with a Role in Binding Adenosine Triphosphate†

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ABSTRACT: An immunoadsorbent specific for the carboxy-terminal sequence -GAPER, which comprises residues 502–506 of the α-polypeptide of ovine sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase], was used to isolate the products of the reaction between the lysine immediately preceding this sequence in the intact protein and either [^{3}H]acetic anhydride or fluorescein 5′-isothiocyanate. Changes in the apparent nucleophilicity of this lysine, Lys_{501}, were observed with both reagents when ATP was bound by the intact, native enzyme poised in the E_1 conformation or when the structure of the enzyme was changed from the E_1 conformation into the E_2–P conformation. With both reagents, a decrease of more than 4-fold in the yield of incorporation occurred during the former change, but a decrease of only 2-fold occurred during the latter. Because a much larger decrease occurred when ATP was bound in the absence of a conformational change than occurred when a major conformational change took place in the absence of the occupation of the active site, these changes in the incorporation of [^{3}H]acetyl suggest that Lys_{501} from the α polypeptide is directly involved in binding ATP within the active site of (Na^+ + K^+)-ATPase. The immunochemical reactions between the specific polyclonal antibodies raised against the sequence -GAPER and denatured or enzymically active (Na^+ + K^+)-ATPase were also investigated. Western blots and the inhibition of enzymic activity caused by the antibody have shown that it can bind to both the denatured and the native form of the α-polypeptide, respectively. The effects of the binding of anti-GAPER immunoglobulins to the native enzyme on the incorporation of fluorescein 5′-isothiocyanate at Lys_{501} demonstrate that they are binding to the native enzyme in the immediate vicinity of Lys_{501}. When an immunoglobulin is bound, the enzyme is inactive.

Sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase],[1] embedded in the plasma membranes of all animal cells, has been extensively studied since Skou (1957) identified its activity in fragments from crab nerves. This enzyme transports potassium into the cell and sodium out of the cell against their concentration gradients (Skou, 1964). Kinetic studies have indicated that (Na^+ + K^+)-ATPase passes through at least four conformations during each turnover. The enzyme can be poised in any one of these four conformations, which are designated as E_1, E_1~P, E_2–P, and E_2, by choosing appropriate concentrations of various substrates or inhibitors (Winslow, 1981).

Fluorescein 5′-isothiocyanate reacts specifically with Lys_{501}, subsequently isolated from the α-polypeptide in the peptide HLLVMK(fluoresceinyl)GAPER, to inactivate the enzyme (Karlish, 1980; Farley et al., 1984).[2] It has also been shown that Lys_{501} is located on the cytoplasmic side of native (Na^+ + K^+)-ATPase (Kyte et al., 1987), which is a protein that spans the plasma membrane and has surfaces exposed to both sides (O'Connell, 1982; Dzhandzhugazyan & Jørgensen, 1985). This lysine residue is located in a sequence conserved among the ATPases responsible for primary active transport (Serrano et al., 1986). This suggests that it is involved in some essential role in the mechanism of the enzyme.

Observations that purport to contradict this suggestion, however, have recently been reported. It has been found that a monoclonal antibody, M8-P1-A3, raised against entire ovine renal (Na^+ + K^+)-ATPase, fortuitously is directed against some subset of the sequence in the peptide, HLLVMKGAPER (Ball & Friedman, 1987), the sequence containing Lys_{501} in the α-polypeptide. This monoclonal antibody also recognized (Na^+ + K^+)-ATPase adsorbed irreversibly to the plastic well of a microtiter plate. When purified (Na^+ + K^+)-ATPase was preincubated with this antibody, its titer for the (Na^+ + K^+)-ATPase adsorbed on the plastic decreased. Unfortunately, 1 μmol of purified (Na^+ + K^+)-ATPase was required to adsorb 10 nmol of antibody in this preincubation. One explanation for this hundredfold disparity would be that only denatured α-polypeptide, which is present unavoidably at a few percent in any purified preparation of (Na^+ + K^+)-ATPase, is antigenic. In any case, antibody is not bound by greater than 95% of the enzyme present in these preincubations, and it is not surprising that it has no effect on enzymic activity.

This paper presents a study of the apparent nucleophilicity of Lys_{501} in its reaction with the small electrophile, acetic anhydride. Changes in its apparent nucleophilicity were ob-

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[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate; Tris, tris(hydroxymethyl)-aminomethane; TTBS, 0.1% Tween-20, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.5.

[2] An incomplete sequence of canine renal (Na^+ + K^+)-ATPase was available (Robert Farley, personal communication). It is identical with the ovine sequence in the region of interest. As this sequence lacked the amino-terminal portions, the numbering of the ovine α polypeptide (Shull et al., 1985) is used here.

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served when ATP binds to the active site and when a major conformational change is induced in the enzyme. The character of these changes clarifies the role of Lys_{501} in the mechanism of $(Na^+ + K^+)$-ATPase. It was found that polyclonal immunoglobulins, raised against the peptide KGAPER, attached as a hapten through its amino-terminal lysine, were able to inhibit $(Na^+ + K^+)$-ATPase activity. It was demonstrated that this resulted from the binding of the antibody to a sequence in the immediate vicinity of Lys_{501}. Taken together, these observations suggest that Lys_{501} is within the active site at which ATP is hydrolyzed.

Experimental Procedures

Materials. [^{3}H]Acetic anhydride (8.0 Ci mmol^{-1}) was purchased from Amersham. Three micromoles of [^{3}H]acetic anhydride was dissolved in 3.0 mL of dry acetonitrile to produce a 1.0 mM solution that was used for additions to samples to be modified. Fluorescein 5′-isothiocyanate was purchased from Molecular Probes Inc.; tris(hydroxymethyl)aminomethane (Tris),[1] Tween-20, diaminobenzidine tetrahydrochloride, soybean trypsin inhibitor, prestained standards for molecular weight, 2-mercaptoethanol, adenosine 5′-triphosphate, ovalbumin, and strophanthidin were from Sigma Chemical Corp.; and trypsin that had been treated with *N*-(*p*-toluenesulfonyl)-L-phenylalanyl chloromethyl ketone was from Worthington Corp. Sodium dodecyl sulfate (NaDodSO$_4$)[1] was purchased from Calbiochem Corp. and recrystallized from 95% ethanol (Burgess, 1969). Imidazole was recrystallized from benzene and then acetone. The subsaturated complex between avidin and biotinylated horseradish peroxidase (ABS reagent) was purchased from Vector Laboratories, Inc. Nitrocellulose membranes were purchased from Schleicher & Schuell. Biotinylated goat antibodies against rabbit γ-globulin were a gift from Dr. Susan S. Taylor. α-Polypeptides and β-polypeptides of porcine $(Na^+ + K^+)$-ATPase were gifts from Dr. Osamu Urayama.

Preparation of $(Na^+ + K^+)$-ATPase. Native $(Na^+ + K^+)$-ATPase was isolated from microsomes prepared from canine kidney (Kyte, 1971). The purified $(Na^+ + K^+)$-ATPase was prepared by the method of Jørgensen (1974), with the modifications of Munson (1981), and assayed for strophanthidin-sensitive ATPase activity in the presence of both sodium and potassium (Kyte, 1971). The specific activity of the enzyme used for these experiments was 800–1000 μmol of P_i mg^{-1} h^{-1}. Concentrations of protein were determined by the method of Lowry et al. (1951) as modified by Kyte (1971) with bovine serum albumin as a standard.

Preparation of the Anti-GAPER Immunoglobulins. Polyclonal antibody raised against the peptide KGAPER, attached as a hapten to serum albumin through its lysine, has been described previously (Kyte et al., 1987). The peptide KGAPER was first synthesized manually by solid-phase techniques (Merrifield, 1963; Merrifield et al., 1982; Stewart & Young, 1969) and then coupled to bovine serum albumin by the glutaraldehyde procedure (Walter et al., 1980). The resulting covalent complex was injected as an antigen into rabbits. Crude precipitates of immunoglobulins from antisera were stored in 50% ammonium sulfate at 4 °C. Precipitates were gathered by centrifugation and dialyzed extensively against phosphate-buffered saline, pH 7.4 at 4 °C.

Antibodies against the sequence -GAPER were purified with an immunoadsorbent made by coupling the synthetic peptide KGAPER to beaded agarose with cyanogen bromide (March et al., 1974). Soluble immunoglobulins were passed at room temperature over a column of this immunoadsorbent. It was washed extensively with phosphate-buffered saline and eluted with 0.2 M glycinium chloride, pH 2.5. The fractions containing eluted immunoglobulin were pooled and dialyzed against phosphate-buffered saline. The antibody used to determine the inhibition of $(Na^+ + K^+)$-ATPase activity was then dialyzed against a buffer containing 1 mM EDTA, 25 mM imidazolium chloride, pH 7.4, to remove phosphate. Concentrations of solutions of immunoglobulins were estimated by the absorbance at 280 nm.

Immunoadsorbent Specific for the Sequence -GAPER. Purified anti-GAPER immunoglobulins were attached to beaded agarose by cyanogen bromide activation to produce an immunoadsorbent (Kyte et al., 1987). The capacity of this immunoadsorbent was determined by saturating it with synthetic peptide, washing with phosphate-buffered saline, eluting the bound peptide with acid, and determining by amino acid analysis the amount of peptide released. The capacity of the immunoadsorbent used for these experiments was 14 nmol in 3 mL of settled agarose.

Labeling of $(Na^+ + K^+)$-ATPase with [^{3}H]Acetic Anhydride. To prepare samples for labeling, purified $(Na^+ + K^+)$-ATPase (15 mg) was centrifuged at 45 000 rpm in a Beckman 50 Ti rotor for 30 min, and the pellet was resuspended in 0.1 M sodium borate, pH 9.2. Reaction with [^{3}H]acetic anhydride was performed at 8–10 °C for 10 min (Kaplan et al., 1971) with three different conditions under which the enzyme assumes the conformations E_1 in the absence of ATP [E_1(–ATP)], E_1 in the presence of ATP [E_1(+ATP)], and E_2–P, respectively. The final concentrations of ovalbumin, $(Na^+ + K^+)$-ATPase, and NaCl in the mixtures, buffered at pH 9.2 with 0.1 M sodium borate, were 0.5 mg mL^{-1}, 4.7 mg mL^{-1}, and 0.1 M, respectively. The final concentration of *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate (CDTA)[1] in the samples poised in the E_1 conformation was 5 mM. The final concentration of ATP in the samples poised in the E_1(+ATP) and E_2–P conformations was 5 mM. The final concentration of $MgCl_2$ in the samples poised in the E_2–P conformation was 20 mM, and the final concentration of strophanthidin was 0.5 mM. The same amount of [^{3}H]acetic anhydride (20 μL containing 20 nmol at a specific radioactivity of 8 Ci mmol^{-1}) was added with rapid mixing to each of the three samples whose final volumes were 1.00 mL. The reaction between protein and acetic anhydride reaches completion under these conditions after 10 min (Kaplan et al., 1971) owing to almost complete hydrolysis of the acetic anhydride. After the reaction, the samples were centrifuged at 45 000 rpm in a Beckman 50 Ti rotor for 60 min to remove unattached [^{3}H]acetic acid, and the pellets were resuspended in 1 mM ethylenediaminetetraacetate (EDTA)[1], 25 mM imidazolium chloride, pH 7.5.

The concentration of enzyme in these reactions (4.7 mg mL^{-1}) is quite high, and it is possible that some of the added ATP has been hydrolyzed. In the situation where the enzyme was poised in the E_1(+ATP) form, no Mg^{2+} was added, and 5 mM CDTA was present to chelate any adventitious Mg^{2+}. Under these conditions, the Mg^{2+}-dependent ATPase activities contaminating the enzyme are completely inhibited. At 37 °C over 10 min, less than 4 mM ATP should be hydrolyzed under these conditions because phosphatases not dependent upon Mg^{2+} are present at specific activities of less than 0.5% that of the fully activated enzyme. The reaction performed here, however, is at 10 °C. With a similar preparation of enzyme at 0.5 mg mL^{-1} in the presence of Mg^{2+} and ATP at 37 °C, only 15% of the 5 mM ATP was hydrolyzed over 30 min (Winslow, 1981). Although the concentration of enzyme was lower in these earlier experiments than in the present

experiments, the temperature was higher, Mg^{2+} was present, and the time interval was longer. On the basis of these considerations, we believe that little ATP is hydrolyzed during the incubation in the presence of CDTA at 10 °C for 10 min and even less during the time in which the [^{3}H]acetic anhydride remains intact. In the situation in which the enzyme was poised in the E_2–P conformation, the MgATP was present only to produce the E_2–P conformation initially which was then trapped by the strophanthidin. Hydrolysis of MgATP following this step is irrelevant.

Labeling of (Na^+ + K^+)-ATPase with Fluorescein 5′-Isothiocyanate. To prepare samples for labeling, purified (Na^+ + K^+)-ATPase (15 mg) was centrifuged at 45 000 rpm in a Beckman Ti 50 rotor for 30 min. The pellet was resuspended in 1.0 mL of 50 mM Tris-HCl, pH 9.2. The reaction with fluorescein 5′-isothiocyanate was performed at a final concentration of 0.10 mM [a molar ratio of 1:5 between (Na^+ + K^+)-ATPase and fluorescein 5′-isothiocyanate] at room temperature for 1.5 h under the same conditions designated E_1(−ATP), E_1(+ATP), and E_2–P as described above and at the same final concentration (4.7 mg mL^{-1}) of enzyme. Samples were then centrifuged at 45 000 rpm for 60 min, and the resulting pellets were resuspended in 1 mM EDTA, 25 mM imidazolium chloride, pH 7.5.

Digestion with Trypsin. Each resuspended, labeled sample was digested with trypsin at a ratio, by weight, of 1:10 between trypsin and ATPase for 3 h at 37 °C, and soybean trypsin inhibitor was added at a 2-fold weight excess over trypsin to stop the reaction (Farley et al., 1984). The mixtures were centrifuged at 45 000 rpm for 1 h. The supernatant was centrifuged again at 45 000 rpm for 30 min and submitted to lyophilization.

Supernatants from tryptic digests of (Na^+ + K^+)-ATPase that had been labeled with either [^{3}H]acetic anhydride or fluorescein 5′-isothiocyanate were passed directly over a column (1.1 cm × 2.7 cm) of the anti-GAPER immunoadsorbent (14-nmol capacity). The liganded immunoadsorbent was washed with phosphate-buffered saline and eluted with 0.1 M sodium phosphate, pH 2.5. Eluted fractions that contained peptides labeled with [^{3}H]acetyl groups were collected, and their tritium content was determined by liquid scintillation. Eluted fractions that contained fluoresceinyl peptides were collected, and the absorbance at 499 nm of each fraction was determined after adjustment of the pH to between 7 and 8. The number of nanomoles of the labeled peptide HLLMVK(fluoresceinyl)GAPER was calculated on the basis of a molar extinction coefficient of 75 000 at pH 8.[3]

Electrophoresis on Polyacrylamide Gels. Samples for electrophoresis were dissolved by adding a small volume of 20% $NaDodSO_4$ (>5 μg of $NaDodSO_4$ for every microgram of protein). The electrophoresis was performed on gels of 5% polyacrylamide by the procedure of Weber and Osborn (1969). Following electrophoresis, the gels were stained with Coomassie brilliant blue and cut into 2-mm slices. The slices were submitted to scintillation counting by the procedure of Drickamer (1976) as modified by Munson (1983).

Immunoblotting. Various amounts of membrane-bound, purified (Na^+ + K^+)-ATPase or its separated polypeptides were dissolved by the addition of $NaDodSO_4$ (>5 μg of $NaDodSO_4$ for each microgram of protein) and separated by electrophoresis on 10% polyacrylamide gels. When the gels were stained for protein, the normal patterns of the α-polypeptides and β-polypeptides were observed. Prestained standard polypeptides were run on one of the lanes of the gel as well. Polypeptides, unstained and prestained, were transferred from the gels onto nitrocellulose membranes by transfer electrophoresis in a buffer containing 20% methanol, 192 mM glycine, and 25 mM Tris (Towbin et al., 1979). After 5 h of transverse electrophoresis, the nitrocellulose membranes were washed for 30 min with gentle agitation in 0.1% Tween-20, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.5 (TTBS),[1] at room temperature. The membranes were transferred to a solution of anti-GAPER immunoglobulins (12 nM in sites specific for -GAPER) and soaked overnight at room temperature with gentle agitation. After an extensive wash with TTBS, the membranes were soaked at room temperature for 1 h with biotinylated goat antibodies against rabbit γ-globulin (Bayer et al., 1979). After the membranes had been washed again with TTBS, they were transferred for 30 min to a solution of avidin to which biotinylated horseradish peroxidase had been attached at subsaturating molar ratios (Hsu et al., 1981). After the membrane was soaked with 500 mM NaCl, 20 mM Tris-HCl, pH 7.5, over 30 min with gentle agitation, it was transferred to a solution for the chromagenic assay of peroxidase (0.1% H_2O_2, 0.05% diaminobenzidine tetrahydrochloride, 0.04% $NiCl_2$, and 0.1 M Tris-HCl, pH 7.2; Hsu & Soban, 1982) for 3–5 s and washed with distilled water over 10 min. The stained nitrocellulose membrane was dried and stored in the dark.

Determination of Inhibition of (Na^+ + K^+)-ATPase by Anti-GAPER Immunoglobulins. Purified (Na^+ + K^+)-ATPase (30 μg, 0.13 nmol) was mixed with varying concentrations of anti-GAPER immunoglobulins or immunoglobulins from the same antiserum from which the anti-GAPER immunoglobulins had been removed by adsorption. The mixtures had a final volume of 1.5 mL and were prepared in 1 mM EDTA, 25 mM imidazolium chloride, pH 7.5. They were allowed to stand overnight at room temperature. Aliquot parts (25 μL) were removed for the determination of strophanthidin-sensitive (Na^+ + K^+)-ATPase activity as described by Kyte (1971).

Inhibition of Labeling by Fluorescein 5′-Isothiocyanate. Purified (Na^+ + K^+)-ATPase (6 mg; 26 nmol) was incubated with anti-GAPER immunoglobulins (4.5 mg; 56 nmol sites) in 4 mL of phosphate-buffered saline, overnight at room temperature, before labeling the enzyme with fluorescein 5′-isothiocyanate. Three otherwise equivalent samples were also preincubated in this way: one that contained 4.5 mg of nonspecific immunoglobulin that had passed through an immunoadsorbent to which KGAPER had been attached; one that had ATP added to it to a final concentration of 3 mM immediately before the labeling reaction but that had been preincubated overnight in phosphate-buffered saline without any antibodies; and one that had been preincubated overnight in phosphate-buffered saline with no further additions. Fluorescein 5-isothiocyanate (280 nmol) was then added as a concentrated solution in dimethyl sulfoxide (10–20 μL) to each sample. Labeling was performed in the dark at pH 7.4 for 1 h. The samples were centrifuged at 45 000 rpm in a Beckmann 50.2 Ti rotor for 1 h; the pellets were resuspended in 1 mM EDTA, 25 mM imidazolium chloride, pH 7.4, and the samples were digested with trypsin. Each supernatant from these digests was split into two parts, one to which 200 nmol of the synthetic peptide KGAPER was added and one to which no addition was made. Each sample was then passed over the immunoadsorbent specific for the sequence -GAPER packed in a column 1.1 cm × 2.7 cm (total capacity of 14 nmol). The immunoadsorbent was washed with phosphate-buffered saline and eluted with 0.1 M sodium phosphate, pH 2.5. The ab-

[3] Myun Han and P. A. George Fortes, personal communication.

Table I: Overall Incorporation of [^{3}H]Acetyl Groups into Polypeptides[a]

conformation of enzyme	area[b] α	area[b] ovalb	cpm[c] α	cpm[c] ovalb	$\frac{cpm_\alpha}{A_{ovalb}}/\frac{cpm_{ovalb}}{A_\alpha}$[d]
E_1(−ATP)	10.9	13.9	5970	8200	0.93
E_1(+ATP)	9.3	13.4	4930	9240	0.76
E_2–P	10.1	14.2	3940	9010	0.62

[a] The results from which this table was constructed are displayed in Figures 1 and 2. [b] From scans of stained gels (Figure 1). [c] From scintillation counting of slices from gels (Figure 2). The counts per minute in slices 9, 10, and 11 were totalled, and a background equal to the mean of the counts per minute in slices 6, 7, and 12 was subtracted. The counts per minute in slices 16, 17, and 18 were totalled, and a background equal to the mean of the counts per minute in slices 15 and 19 was subtracted. [d] Specific radioactivity of α-polypeptide (α) normalized to specific radioactivity of ovalbumin (ovalb).

sorbance at 499 nm of each fraction of 1 mL was determined after adjusting the pH to 7–8. From the molar concentrations, using an extinction coefficient[3] of 75 000, the numbers of nanomoles of the peptide HLLVMK(fluoresceinyl)GAPER (Farley et al., 1984) were calculated.

Results

Labeling of (Na^+ + K^+)-ATPase with [^{3}H]Acetic Anhydride. Acetic anhydride is an active electrophile that can react with nucleophilic amino acids. Under the conditions employed in these experiments, it probably irreversibly modifies only lysines in the protein (Kaplan et al., 1971) by forming the corresponding *N*-alkyl[^{3}H]acetamides. Acetic anhydride completely and irreversibly inactivates (Na^+ + K^+)-ATPase at final concentrations of 2 mM or greater and irreversibly inactivates the enzyme by 80% at a final concentration of 0.5 mM. In all of the experiments to be described here, it was used at concentrations of less than 30 μM, in its radioactive form. At these concentrations, negligible irreversible inhibition occurs, and the reagent is only being used as a probe of the apparent nucleophilicity of particular lysines (Kaplan et al., 1971). Under these circumstances, in which water is competing with lysines on the proteins and the concentration of lysine is low, the yield of incorporation, at a constant pH and the same initial concentration of acetic anhydride, is directly proportional to the second-order rate constant for the reaction between the lysine and the electrophile. Because it is a small uncomplicated electrophile, in contrast to fluorescein 5′-isothiocyanate, acetic anhydride should be reacting in very low yield with all accessible lysines anywhere in the protein.

The effects of either the binding of the substrate ATP in the absence of a global conformational change or one of the major changes in the conformation of the enzyme on the overall incorporation of [^{3}H]acetyl groups into the α-polypeptide of native (Na^+ + K^+)-ATPase were examined. Ovalbumin was included in the reactions to serve as an internal standard because its reaction with [^{3}H]acetic anhydride should not be, and was not noticeably (Table I), affected by either ATP, $MgCl_2$, or strophanthidin. The results are presented as the specific incorporation of [^{3}H]acetic anhydride into the α-polypeptide relative to the specific incorporation into ovalbumin under the conditions of reaction examined (Table I). No significant changes in the relative amounts of α-polypeptide and ovalbumin were observed on polyacrylamide gels of the samples (Figure 1A–C), and areas from scans such as these and counts per minute in slices from such gels (Figure 2) were used to calculate the specific radioactivities (Table I).

When (Na^+ + K^+)-ATPase was poised in the E_1 conformation by suspending it in a solution containing 0.1 M NaCl and 5 mM CDTA (Jørgensen, 1975; Castro & Farley, 1979; Karlish, 1980), the addition of ATP caused a reproducible decrease in the overall incorporation of [^{3}H]acetyl groups into the entire α-polypeptide (Figure 2, Table I). When ATP, Mg^{2+}, and strophanthidin are added to (Na^+ + K^+)-ATPase, the enzyme changes its conformation dramatically and assumes the E_2–P conformation (Siegel et al., 1969). A further decrease in incorporation of [^{3}H]acetyl groups was observed under these conditions (Figure 2, Table I).

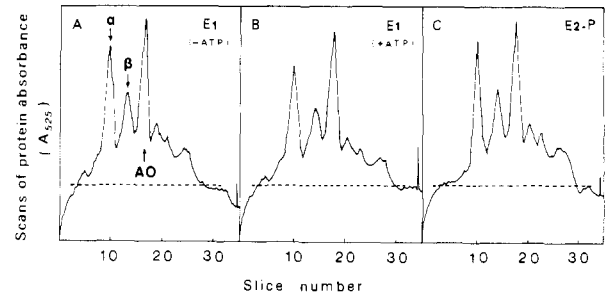

FIGURE 1: Scans of polyacrylamide gels on which samples modified with [^{3}H]acetic anhydride were run. Reactions were initiated by adding equal portions of (Na^+ + K^+)-ATPase (36 μg suspended in 0.1 M sodium borate, pH 9.2) to 0.1-mL solutions at 10 °C composed such that the final concentrations were 0.5 mg mL^{-1} ovalbumin, 0.1 M NaCl, and 0.1 M sodium borate, pH 9.2. In samples poised in the E_1 conformation in the absence of ATP (A), CDTA was added to a concentration of 5 mM; in samples poised in the E_1 conformation in the presence of ATP (B), CDTA was added as well as ATP to 5 mM; in samples poised in the E_2–P conformation (C), ATP, $MgCl_2$, and strophanthidin were added to concentrations of 5, 20, and 1 mM, respectively. [^{3}H]Acetic anhydride (3 μL containing 3 nmol at a specific radioactivity of 8 Ci $nmol^{-1}$) was added to each sample. After a reaction of 10 min, the samples were submitted directly to electrophoresis on polyacrylamide gels run in 0.1% $NaDodSO_4$. The gels were stained, clarified, and then scanned for absorbance at 525 nm. Positions of the α-polypeptide (α) and the β-polypeptide (β) of (Na^+ + K^+)-ATPase and ovalbumin (AO) are indicated on panel A. The base lines used to calculate areas have been added to each tracing.

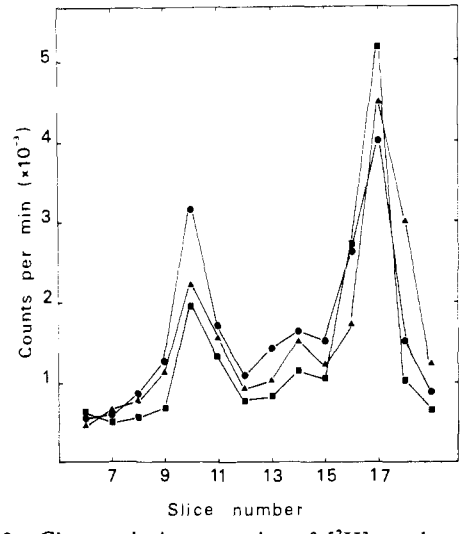

FIGURE 2: Changes in incorporation of [^{3}H]acetyl groups from [^{3}H]acetic anhydride into the entire α-polypeptide of native (Na^+ + K^+)-ATPase caused by the binding of ATP to the enzyme poised in the E_1 conformation or the shifting of the conformation from E_1 to E_2–P. The radioactivity associated with each of the polypeptides was determined by measuring the cpm tritium in each slice of 2 mm from the stained gels whose scans are displayed in Figure 1. The abscissas in Figures 1 and 2 are provided with scales indicating the positions of the slices. Symbols used are (●) E_1(−ATP), (▲) E_1(+ATP), and (■) E_2–P.

Determination of the Incorporation of [^{3}H]Acetyl Groups and Fluoresceinyl Groups into Lys_{501} in the α-Polypeptide of Native (Na^+ + K^+)-ATPase. (Na^+ + K^+)-Adenosinetri-

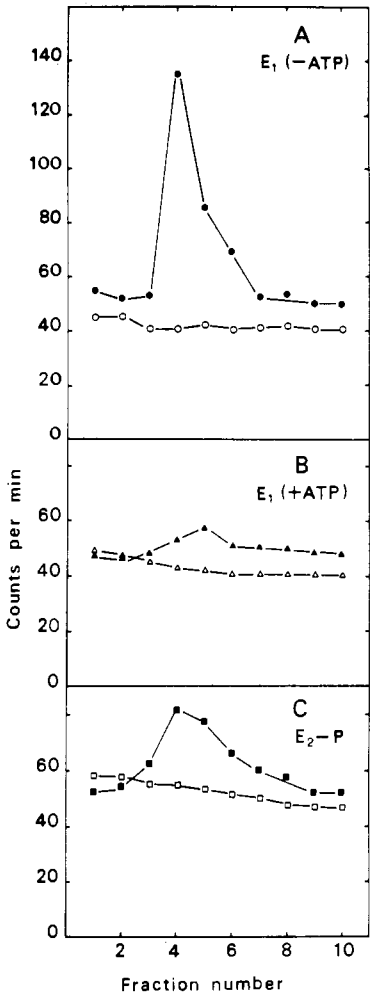

FIGURE 3: Immunoadsorption of tryptic digests of $(Na^+ + K^+)$-ATPase labeled with [^{3}H]acetic anhydride under the conditions designated: (A) E_1(−ATP); (B) E_1(+ATP); (C) E_2–P. Supernatants of tryptic digests of labeled native enzyme were either added directly (closed symbols) to a column (3 mL) of beaded agarose to which anti-GAPER immunoglobulin had been attached (14-nmol capacity) or added after they had been mixed (open symbols) with 400 nmol of the synthetic peptide KGAPER. The column was then washed with 15 mL of phosphate-buffered saline and eluted with 0.1 M sodium phosphate, pH 2.5. Only the acidic elutions are displayed, and the pH was dropped at fraction 1. Each fraction (1.0 mL) was collected and submitted to liquid scintillation.

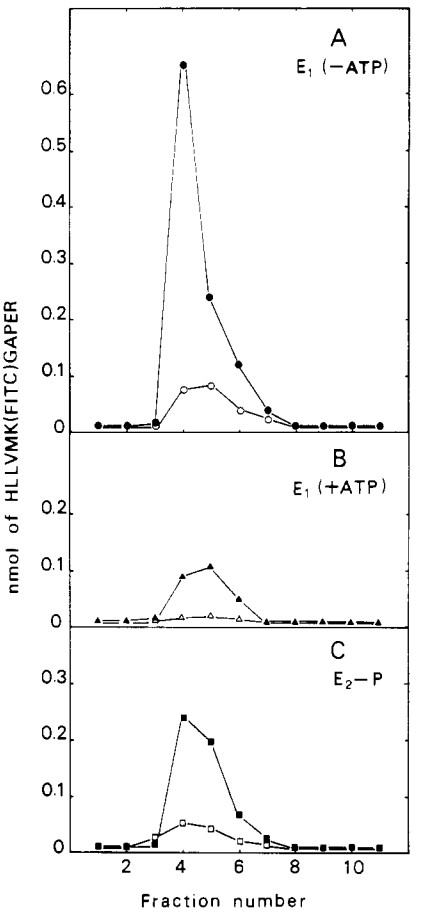

FIGURE 4: Immunoadsorption of tryptic digests of $(Na^+ + K^+)$-ATPase labeled with fluorescein 5′-isothiocyanate under the conditions designated: (A) E_1(−ATP); (B) E_1(+ATP); (C) E_2–P. Supernatants of tryptic digests were either added directly (closed symbols) to a column (3 mL) of beaded agarose to which anti-GAPER immunoglobulin had been attached (14-nmol capacity) or added after they had been mixed (open symbols) with 400 nmol of the synthetic peptide KGAPER. Fractions from the immunoadsorbent which contained HLLVMK(fluoresceinyl)GAPER were collected, and the absorbance at 499 nm of each fraction, after adjustment to pH 7–8, was determined. The number of nanomoles of fluoresceinyl peptide was calculated, on the basis of an extinction coefficient of 75 000.

phosphatase was modified with [^{3}H]acetic anhydride under the same three conditions used for the samples described in Figure 2 and Table I. The enzyme was also modified with fluorescein 5′-isothiocyanate in three, respective, duplicate samples. Following the labeling reactions, all six samples were digested in their native state with trypsin. Each digest should contain the modified peptides HLLVMK(X)GAPER, where X is either an [^{3}H]acetyl group or a fluorescein 5′-isothiocyanyl group attached to Lys_{501} of the α-polypeptide (Farley et al., 1984).

These digests were passed over an immunoadsorbent directed against the carboxy-terminal sequence -GAPER (Kyte et al., 1987). Adsorbed tritium (Figure 3) or fluorescein (Figure 4) was eluted in each case with acid. As a control, to elucidate the fraction of the bound and eluted tritium or fluorescein attached to tryptic peptides with the carboxy-terminal sequence -GAPER, equivalent samples from each digest were mixed with a large molar excess of the synthetic peptide KGAPER before immunoadsorption. Very little tritium or fluorescein, respectively, was adsorbed and eluted in these control experiments (Figures 3 and 4, respectively). In all cases, the same relative amount of each digest, based on the protein concentration of the original sample of $(Na^+ + K^+)$-ATPase, was passed over the column of immunoadsorbent. The nanomoles of $(Na^+ + K^+)$-ATPase that contributed to each sample added to the immunoadsorbent (<10 nmol) was purposely chosen to be less than the capacity of the column for the synthetic peptide KGAPER.

When ATP was added to $(Na^+ + K^+)$-ATPase, poised in the E_1 conformation, incorporation of both [^{3}H]acetyl groups and fluorescein 5′-thioureidyl groups into Lys_{501} in its α-polypeptide was dramatically reduced (Figures 3A,B and 4A,B, respectively, and Table II). The decreases in incorporation seen with each reagent were similar: a decrease of 83% in the case of modification by [^{3}H]acetic anhydride and a decrease of 75% in the case of modification by fluorescein 5′-isothiocyanate. When the enzyme was shifted into the E_2–P conformation, in which the largest decrease in the overall incorporation of [^{3}H]acetyl groups into all of the lysines of the enzyme was seen (Figure 2, Table I), much less of a decrease in incorporation of [^{3}H]acetic anhydride into Lys_{501} occurred (Figure 3C and Table II) than was seen with only the binding of ATP (Figure 3B and Table II). The same behavior was observed with the incorporation of fluorescein 5′-isothiocyanyl groups (Figure 4B and Figure 4C, respectively,

Table II: Incorporation of Electrophiles into Lys_{501}

expt	incorporation (%)[a]	
	[^{3}H]acetyl	fluorescein 5′-isothiocyanate
	E_1(+ATP)	
1	22	30
2	15	20
3	19	26
4	13	24
	E_2–P	
1	54	46
2	60	52
3	52	50
4	56	48

[a] Data were from four experiments identical with the ones described in Figures 3 and 4. Calculations based on cpm eluted with acid in experimental runs minus cpm eluted with acid in control runs. The conformation E_1(−ATP) was chosen as the reference conformation for 100% incorporation.

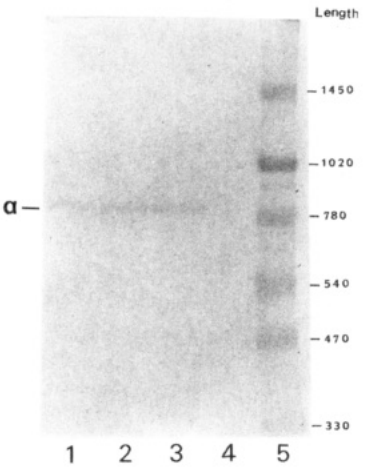

FIGURE 5: Binding of polyclonal anti-GAPER immunoglobulins to the α-polypeptide of (Na^+ + K^+)-ATPase in Western transfer analysis. Samples of purified (Na^+ + K^+)-ATPase, dissolved by the addition of $NaDodSO_4$, were separated by electrophoresis on a gel of 10% polyacrylamide. The separated polypeptides were then transferred to nitrocellulose membranes and reacted with polyclonal rabbit immunoglobulins directed against the sequence -GAPER followed by goat antibodies against rabbit γ-globulin to which horseradish peroxidase was then attached. The distribution of bound antibody was determined by peroxidase-dependent staining. Lane 1, canine (Na^+ + K^+)-ATPase (3 μg). Lane 2, canine (Na^+ + K^+)-ATPase (5 μg). Lane 3, α-polypeptide from porcine (Na^+ + K^+)-ATPase. Lane 4, β-polypeptide of porcine (Na^+ + K^+)-ATPase. Lane 5, standard polypeptides: α_2-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, and lactate dehydrogenase.

and Table II). Again, the decreases in the incorporation of tritium and fluoresceinyl were quite similar: one of 44% in the case of modification by [^{3}H]acetic anhydride and one of 51% in the case of modification by fluorescein 5′-isothiocyanate. These decreases in the incorporation into Lys_{501} can be compared with the 31% decrease in the overall incorporation under these conditions (Table I).

Anti-GAPER Immunoglobulins Bind to the α-Polypeptide of (Na^+ + K^+)-ATPase. Antibodies raised against the hapten -GAPER and purified by immunoadsorption stain the intact α-polypeptide of (Na^+ + K^+)-ATPase that has been transferred to nitrocellulose membranes from a polyacrylamide gel by Western blotting (Figure 5). Both canine and porcine α-polypeptides are recognized by the anti-GAPER immunoglobulins, but porcine β-polypeptide is not. These results demonstrate that anti-GAPER immunoglobulins bind to the sequence -GAPER- when it is in the middle of an intact polypeptide.

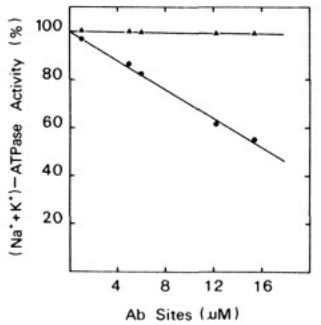

FIGURE 6: Inhibition of (Na^+ + K^+)-ATPase by anti-GAPER immunoglobulins. Purified (Na^+ + K^+)-ATPase (30 μg, 0.13 nmol in each sample) was preincubated in a final volume of 1.5 mL containing a 1.0, 5, 6, 12, or 15 μM sample of sites of either anti-GAPER immunoglobulins (●) or the nonspecific immunoglobulins that had passed through an immunoadsorbent to which the synthetic peptide KGAPER had been attached (▲). Following an overnight preincubation, samples were assayed for (Na^+ + K^+)-ATPase activity. The numbers presented are averages from three separate experiments.

Anti-GAPER Immunoglobulins Inhibit the Enzymic Activity of (Na^+ + K^+)-ATPase. At a relatively high molar concentration of sites, 10–20 μM, anti-GAPER immunoglobulins were able to inhibit (Na^+ + K^+)-ATPase by binding to the enzyme during a preincubation in which the concentration of (Na^+ + K^+)-ATPase was about 1 μM (Figure 6). Excess antibody was needed to see inhibition; at a molar ratio of 20 nmol of antibody sites for each nanomole of enzyme, inhibition was 50% (Figure 6).

During the purification of anti-GAPER immunoglobulins, the redissolved protein that has been precipitated from serum by 50% ammonium sulfate is passed over an immunoadsorbent to which the peptide KGAPER has been attached to remove the specific immunoglobulins. The crude nonspecific immunoglobulins that had passed through this immunoadsorbent did not inhibit the enzyme when they were preincubated with it at the same concentrations as the specific immunoglobulins (Figure 6). Because these control immunoglobulins were those that passed through the immunoadsorbent during the purification of the anti-GAPER immunoglobulins themselves, they should have contained any nonspecific antibodies that contaminated the anti-GAPER immunoglobulins.

Competition between Anti-GAPER Immunoglobulins and Labeling with Fluorescein 5′-Isothiocyanate. Anti-GAPER immunoglobulins, bound to native (Na^+ + K^+)-ATPase during a preincubation, decreased the subsequent incorporation of fluorescein 5′-isothiocyanate into Lys_{501} of the α-polypeptide (Figure 7C) while nonspecific immunoglobulins did not (Figure 7B). That the fluorescence bound and released from the immunoadsorbent was on Lys_{501} is supported by the fact that the addition of ATP just prior to the reaction with fluorescein 5′-isothiocyanate markedly decreased the yield of eluted fluorescein (Figure 7D). The significant decrease in immunoadsorption that occurs when synthetic KGAPER is mixed with the samples for the immunoadsorbent (Figure 7) also demonstrates that the eluted fluorescein was attached to a peptide with the carboxy-terminal sequence -GAPER.

In the particular experiment described in Figure 7C, (Na^+ + K^+)-ATPase and anti-GAPER immunoglobulins were mixed at a ratio of 2.2 nmol of sites to every nanomole of enzyme, and a 30% decrease in the bound and eluted fluorescein was observed. At a ratio of 2.2 nmol of sites to every nanomole of (Na^+ + K^+)-ATPase, only a 6% decrease in enzymic activity was seen in the experiment described in Figure 6. At least two factors could be responsible for this difference. First, for technical reasons, aliquot parts had to be removed

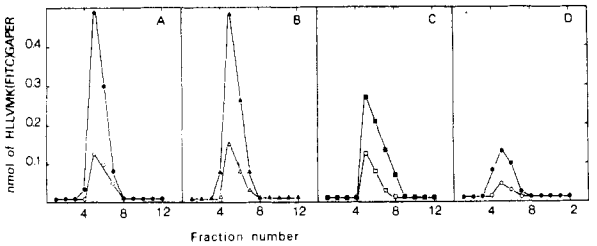

FIGURE 7: Competition of anti-GAPER antibody with fluorescein 5′-isothiocyanate for Lys_{501} from the α-polypeptide of native (Na^+ + K^+)-ATPase. Enzyme (26 nmol in each sample of final volume 4 mL) was modifed with fluorescein 5′-isothiocyanate (280 nmol in each sample) after it had been preincubated with antibodies or had been mixed with ATP immediately before the labeling. The labeled (Na^+ + K^+)-ATPase was collected by centrifugation and digested with trypsin in its native state to release the peptide HLLVMK-(fluoresceinyl)GAPER. Aliquot parts of supernatants from the tryptic digests were either added directly (closed symbols) to a column (3 mL) of beaded agarose to which anti-GAPER immunoglobulins had been attached (14-nmol capacity) or added after they had been mixed with 200 nmol of the synthetic peptide KGAPER (open symbols). The immunoadsorbents were washed with phosphate-buffered saline and eluted with 0.1 M sodium phosphate, pH 2.7. Fluorescein (nanomoles) in each fraction was determined by asorbance after adjusting the pH to 7–8. (A) Enzyme labeled in absence of additions; (B) enzyme labeled after preincubation in a final volume of 4 mL with 50 nmol of sites of nonspecific antibody (antibody not adsorbed by an immunoadsorbent to which KGAPER had been attached); (C) enzyme labeled after preincubation in a final volume of 4 mL with 56 nmol of sites of anti-GAPER immunoglobulins; (D) enzyme labeled after addition of ATP to final concentration of 3 mM immediately before addition of fluorescein 5′-isothiocyanate.

from preincubations and diluted 20-fold into assay solutions to measure enzymic activity (Figure 6). After this dilution, bound immunoglobulin may have dissociated from the enzyme, and this would have caused inhibition to decrease. Second, the total molar concentration of anti-GAPER immunoglobulins in the preincubation of the experiment described in Figure 7C was 14 μM in sites, and the concentration of free immunoglobulin was probably around 10 μM in sites. At this free concentration, the inhibition of (Na^+ + K^+)-ATPase activity in the experiment described in Figure 6 would have been 30%.

Whether the molar ratio of antigen to antibody or the free concentration of antibody is the critical variable cannot be decided unambiguously. If free concentration were the only influential variable, then inhibition of enzymic activity would be the same as inhibition of modification by fluorescein 5′-isothiocyanate under equivalent conditions. If molar ratios were the only influential variable, then inhibition of modification would be greater than inhibition of enzymic activity under equivalent conditions.

Discussion

Conformational changes of (Na^+ + K^+)-ATPase can be monitored by following the incorporation of [^{3}H]acetyl groups into the entire α-polypeptide of the native enzyme (Figures 1 and 2). In the change from the E_1 conformation to the E_2–P conformation, there is a 31% decrease in the mean second-order rate constant (Kaplan et al., 1971) between acetic anhydride and those amino acids, mostly lysines, that react irreversibly with acetic anhydride (Table I). This suggests that a rather remarkable change in the conformation of the protein has occurred during this transition. Although the inactivation of enzymic activity by acetic anhydride has been used as an indicator of the effects of various ligands on (Na^+ + K^+)-ATPase (Robinson & Flashner, 1979), this is the first time that the rate of incorporation of a small, widely promiscuous molecule like acetic anhydride into the entire enzyme has been used to sense the conformational changes of native (Na^+ + K^+)-ATPase.

It is possible that these changes in nucleophilicity result from more than just a change in conformation. When ATP is added to the enzyme, there is also a significant decrease in overall nucleophilicity (Table I). This could be due to the fact that ATP is a trianion or tetraanion and several of the 50 lysines in the α-polypeptide are in the site to which it binds. These lysines could become shielded from reaction with acetic anhydride upon the binding of ATP. If they were particularly nucleophilic under the conditions chosen for the labeling reaction, an elimination of incorporation into these lysines could explain the 18% decrease in overall incorporation of [^{3}H]acetyl caused by ATP (Table I). It is doubtful, however, that the binding site for strophanthidin, because it is so hydrophobic, contains lysines that are masked when it is bound or that the lone phosphate present on the enzyme in the E_2–P conformation would tie up many lysines, so the even larger decrease seen during the shift between the E_1 conformation and the E_2–P conformation (Table I) probably does not result from shielding of a set of lysines but rather the change in conformation.

The decrease in overall incorporation of [^{3}H]acetic anhydride into the α-polypeptide of (Na^+ + K^+)-ATPase observed when ATP is added to the enzyme in a solution containing only sodium could, however, also result from a change in conformation. In the presence of only Na^+, (Na^+ + K^+)-ATPase is supposed to be in the E_1 conformation (Jørgensen, 1975; Castro & Farley, 1979; Karlish, 1980), and the addition of ATP in the absence of Mg^{2+} is not supposed to change the conformation (Jørgensen, 1975; Castro & Farley, 1979). It was on this basis that these experiments were designed. It is possible, however, that a conformational change does occur. For example, Na^+ in the absence of ATP may not be sufficient to pull all of the enzyme into the E_1 conformation under certain circumstances, and the addition of ATP, which acts synergistically with Na^+ to change the conformation (Mårdh & Post, 1977), may complete the transformation. From the rate of inactivation of (Na^+ + K^+)-ATPase by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, it has been argued that (Na^+ + K^+)-ATPase has a different conformation in the presence of ATP alone from that in the presence of Na^+ alone (Pedemonte & Kaplan, 1986). Unfortunately, this inference was based on the fact that ATP had no effect on the modification, a negative result, while Na^+, K^+, Mg^{2+}, Tris-HCl, and choline chloride each separately accelerated the reaction, presumably by an ionic strength effect. It would be fair to say that these latter results are equivocal. As ATP is such a large substrate, it may be difficult to separate local changes in the active site resulting simply from its binding from global conformational changes occurring subsequent to its binding.

Under the conditions used for incorporating [^{3}H]acetic anhydride into (Na^+ + K^+)-ATPase, the yield of incorporation into a given lysine residue in the protein is directly proportional to the numerical value of the second-order rate constant for the reaction between acetic anhydride and that lysine (Kaplan et al., 1971). Therefore, relative yields of incorporation will be referred to as relative rates of incorporation. The changes in the rates of incorporation of [^{3}H]acetyl suggest that Lys_{501} from the α-polypeptide is involved in binding ATP within the active site of (Na^+ + K^+)-ATPase. When ATP binds to the enzyme already in the E_1 conformation, the second-order rate constant of the reaction of Lys_{501} with [^{3}H]acetic anhydride decreases 6-fold (Table II). If the only difference that occurs when ATP, in the absence of Mg^{2+}, is added to (Na^+ +

K^+)-ATPase in the E_1 conformation is the occupation of the active site, then a reasonable explanation of this result would be that Lys_{501} is one of the amino acid residues in the active site that directly associates with the substrate, ATP.

It was possible that the decrease in the nucleophilicity of Lys_{501} when ATP binds was due solely to a change in the conformation of the enzyme. When, however, one of the largest conformational changes of the enzyme previously reported, that between E_1 and E_2–P, was induced, a much smaller decrease in incorporation of [^{3}H]acetyl groups into Lys_{501} (less than 2-fold, Table II) occurred. This smaller decrease in incorporation was of about the same magnitude as the one that occurred over the whole protein during this same conformational change (Table I). Even this smaller decrease in incorporation of [^{3}H]acetyl into Lys_{501}, observed when the E_2–P conformation is produced by phosphorylation with MgATP in the presence of Na^+ and stabilized by the binding of strophanthidin, may be due to the binding of ATP to the active site rather than the change in the conformation of the enzyme. When the E_2–P conformation has ouabain bound to it, ATP will not bind to the active site with high affinity (Hegyvary & Post, 1971), but ATP is present in the solution used in the present experiments at a concentration high enough (5 mM) to permit it to bind to the active site with low affinity.

There are kinetic observations consistent with the possibility that ATP can bind to the active site in the E_2–P–ouabain complex with low affinity. When the E_2–P–[^{3}H]ouabain complex is isolated by dilution and centrifugation from the solution in which it was formed, [^{3}H]ouabain dissociates from it at a rate that nevertheless depends on how it was formed (Yoda & Yoda, 1974; Askari et al., 1988). If the complex was formed in the presence of MgATP and Na^+ (type I), the [^{3}H]ouabain dissociates more rapidly than it does if the complex was formed in the presence of Mg^{2+} and $HOPO_3^{2-}$ (type II). Addition of ATP or Na^+ or both to the solution in which dissociation is occurring can shift the type II complex into a type I complex and in this way hasten the dissociation of [^{3}H]ouabain. This is, however, a kinetic phenomenon, which does not require continuous binding of the ATP to the E_2–P–[^{3}H]ouabain complex, because once the type I complex is formed it will dissociate rapidly even in the absence of ATP. That the effect of ATP on the dissociation of ouabain is kinetic in nature rather than an equilibrium effect associated with continuous binding is illustrated by the fact that if dissociation of [^{3}H]ouabain, bound to (Na^+ + K^+)-ATPase, is measured by adding excess unradioactive ouabain, rather than by the removal of [^{3}H]ouabain by dilution and centrifugation, no effect of ATP on the rate of dissociation is observed, regardless of how the complex was initially formed (Wallick et al., 1980; Forbush, 1983). This latter situation more closely resembles the present circumstances, in which strophanthidin is present continuously, and these results suggest that ATP cannot bind to the enzyme under the conditions that were chosen.

The addition of ATP to a solution containing the E_2–P–[^{3}H]ouabain complex at equilibrium in the presence of Mg^{2+}, $HOPO_3^{2-}$, and subsaturating levels of [^{3}H]ouabain can decrease the amount of bound [^{3}H]ouabain to the level that would have prevailed had ATP been present initially (Tobin et al., 1974). When either the E_2–^{32}P conformation is produced from Mg^{2+} and $HO^{32}PO_3^{2-}$ or the E_2–^{32}P–ouabain complex is produced from Mg^{2+}, $HO^{32}PO_3^{2-}$, and ouabain, their rates of spontaneous dephosphorylation are decreased 2-fold by the addition of ATP (Askari & Huang, 1982). All of these results suggest that ATP can bind to these forms of the enzyme. All of these alterations, however, are kinetic effects of ATP that do not require that it be bound continuously or even for a significant fraction of time to the E_2–P conformation of the E_2–P–ouabain complex.

Finally, there are complicated, cooperative, and antagonistic effects of ATP and $HOPO_3^{2+}$ on either the release of occluded $^{86}Rb^+$ from (Na^+ + K^+)-ATPase (Forbush, 1987) or the exchange of Rb^+ for Rb^+ catalyzed by (Na^+ + K^+)-ATPase (Karlish et al., 1982) that have required an explanation. One of the complexes included in kinetic mechanisms invoked to explain the kinetic behavior observed in these experiments is a complex between ATP and E_2–P. Whether or not there are kinetic mechanisms that can provide satisfactory explanations of the data without including such a complex is not known.

If ATP can bind to the active site of the E_2–P conformation when it is stabilized by the binding of strophanthidin, then the decrease in the nucleophilicity of Lys_{501} seen under these circumstances could be due to both the binding of nucleotide and the change in conformation, and the change in conformation would be even less important in determining the nucleophilicity of Lys_{501} than it seems to be. Therefore, it can be concluded that Lys_{501} responds more to the binding of ATP than to any changes in conformation that ATP might elicit.

The same behavior is seen with fluorescein 5′-isothiocyanate as with [^{3}H]acetic anhydride. That ATP prevents the cyanylation of Lys_{501} by this electrophile has already been shown (Farley et al., 1984), but there were two ambiguities in this result. First, it was possible that the protection afforded by ATP was due to a change in the conformation of the enzyme. The change that occurs when the E_1 conformation of the enzyme is converted to the E_2–P conformation with strophanthidin bound to it is one of the most extreme to which the enzyme can be submitted (Winslow, 1981). The incorporation of fluorescein 5′-isothiocyanate into Lys_{501}, however, decreases by only 2-fold when the enzyme is transferred from the E_1 conformation to the E_2–P conformation (Table II), a change similar to the 31% decrease that occurs in the overall incorporation of acetic anhydride into the entire enzyme (Table I). These results suggest that the far more substantial effect of ATP on the incorporation of this reagent into Lys_{501} is not due to a change in the conformation of the enzyme.

Second, it is possible, indeed it has been concluded from some of the previous results (Farley et al., 1984), that fluorescein 5′-isothiocyanate binds to the enzyme before it incorporates covalently. This seems to be the only way to explain its peculiar selectivity for only one or two lysines. If this is the case, changes in only the binding of fluorescein 5′-isothiocyanate, which should determine whether or not it is juxtaposed to Lys_{501}, could have explained the protection afforded by ATP to its incorporation, and there would be no reason to assume that Lys_{501} is directly involved in the binding of ATP. It is unlikely that acetic anhydride can bind to the enzyme. Yet the incorporation of [^{3}H]acetic anhydride into Lys_{501} changes in concert with the incorporation of fluorescein 5′-isothiocyanate. These results demonstrate that changes in the nucleophilicity of Lys_{501} are sufficient to explain the apparent protection afforded by ATP, and no argument requiring competition between fluorescein 5′-isothiocyanate and ATP for the active site need be invoked. Nevertheless, the possibility that Lys_{501} is directly involved in the binding of ATP suggests that fluorescein 5′-isothiocyanate binds to the enzyme at a location near the binding site for ATP. As this reagent does not resemble ATP, this juxtaposition is probably fortuitous.

All of these results demonstrate that Lys_{501} is intimately involved in the catalysis performed by (Na^+ + K^+)-ATPase.

It comes as no surprise that antibodies directed against the sequence -GAPER can inhibit the enzymatic activity (Figure 6) by binding to this sequence in the native enzyme (Figure 7).

In contrast to these results with polyclonal antibodies, however, a monoclonal antibody directed against a subset of the sequence, HLLVMKGAPER, has been described that was unable to inhibit (Na^+ + K^+)-ATPase activity (Ball & Friedman, 1987), presumably because it could not bind to the native enzyme. One of the disadvantages of monoclonal antibodies is that only one site for antigen with a unique specificity is present in the population. If an antigenic sequence is difficult to approach in the native enzyme, a monoclonal antibody that recognizes that sequence in a denatured accessible form has a good chance of failing to recognize the same sequence in the native protein. A population of polyclonal immunoglobulins has many different sites for antigen within it and has many opportunities to discover the antigenic sequence. In the present case, one or more of the unique antibodies in the polyclonal collection of anti-GAPER immunoglobulins has been able to bind to the antigenic sequence in native, enzymically active (Na^+ + K^+)-ATPase. These competent antibodies represent between 3% and 10% of the population (Figures 6 and 7).

An inference can be drawn from a comparison of the present results (Figures 6 and 7) and those of Ball and Friedman (1987). The monoclonal antibody raised by Ball and Friedman against (Na^+ + K^+)-ATPase recognizes some subset of the sequence -HLLVMKGAPER- when it is in a small water-soluble peptide but not when it is in native (Na^+ + K^+)-ATPase. The small fraction of the enzyme that it does recognize as antigenic (<5%) is probably denatured and unfolded. It was probably denatured unfolded (Na^+ + K^+)-ATPase in the injected antigen that elicited the production of this particular monoclonal antibody.

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Registry No. ATPase, 9000-83-3; Lys, 56-87-1; ATP, 56-65-5; acetic anhydride, 108-24-7.

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