Uncoupling of ATP Binding to Na^+,K^+-ATPase from Its Stimulation of Ouabain Binding: Studies of the Inhibition of Na^+,K^+-ATPase by a Monoclonal Antibody†

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ABSTRACT: The effects of a monoclonal antibody, prepared against the purified lamb kidney Na^+,K^+-ATPase, on the enzyme's Na^+,K^+-dependent ATPase activity were analyzed. This antibody, designated M10-P5-C11, is directed against the catalytic subunit of the "native" holoenzyme. It inhibits greater than 90% of the ATPase activity and acts as a noncompetitive or mixed inhibitor with respect to the ATP, Na^+, and K^+ dependence of enzyme activity. It inhibits the Na^+- and Mg^{2+}ATP-dependent phosphoenzyme intermediate formation. In contrast, it has no effect on K^+-dependent *p*-nitrophenylphosphatase (pNPPase) activity, the interconversion of the phosphoenzyme intermediates, and ADP-sensitive or K^+-dependent dephosphorylation. It does not alter ATP binding to the enzyme nor the covalent labeling of the enzyme at the presumed ATP site by fluorescein 5′-isothiocyanate (FITC), but it prevents the ATP-induced stimulation in the rate of cardiac glycoside [^{3}H]ouabain binding to the Na^+,K^+-ATPase. M10-P5-C11 binding appears to inhibit enzyme function by blocking the transfer of the γ-phosphoryl of ATP to the phosphorylation site after ATP binding to the enzyme has occurred. In the presence of Mg^{2+}ATP, it also prevents the ATP-induced transmembrane conformational change that enhances cardiac glycoside binding. This uncoupling of ATP binding from its stimulation of ouabain binding and enzyme phosphorylation demonstrates the existence of an enzyme–Mg^{2+}ATP transitional intermediate preceding the formation of the Na^+-dependent ADP-sensitive phosphoenzyme intermediate. These results are also consistent with a model of the Na^+,K^+-ATPase active site being composed of two distinct but interacting regions, the ATP binding site and the phosphorylation site.

The plasma membrane Na^+,K^+-ATPase regulates the active transport of Na^+ and K^+ across the cell membrane, and it is the pharmacological receptor for cardiac glycosides. It is composed of two subunits, the α subunit ($\sim$100 000 daltons), which contains the ATP binding site, the phosphorylation site, and the cardiac glycoside receptor, and the β subunit ($\sim$50 000 daltons), which is a glycoprotein with no clear functional role [see reviews by Glynn and Karlish (1975), Stekhoven and Bonting (1981), and Jorgensen (1982)]. The generally accepted reaction scheme for the turnover cycle is as follows (Fahn et al., 1966; Post et al., 1969; Mardh & Lindahl, 1977):

$$E_1 + Mg^{2+}ATP \underset{Na^+}{\overset{Na^+}{\rightleftharpoons}} Na^+ \cdot Mg \cdot E_1 \sim P + ADP \quad (1)$$

$$Mg^{2+} \cdot E_1 \sim P \cdot Na^+ \underset{Na^+}{\overset{Na^+}{\rightleftharpoons}} Mg^{2+} \cdot E_2\text{-}P \quad (2)$$

$$Mg^{2+} \cdot E_2\text{-}P \underset{K^+}{\overset{K^+}{\rightleftharpoons}} K^+ \cdot E_2 + Mg^{2+} + P_i \quad (3)$$

$$K^+ \cdot E_2 \underset{Na^+}{\overset{Na^+}{\rightleftharpoons}} Na^+ \cdot E_1 \quad (4)$$

The enzyme undergoes a series of ligand-induced conformational changes during the reaction cycle, which include the formation of Na^+ and K^+ forms of the enzyme and at least two different phosphoenzyme intermediates (Jorgensen, 1982). In addition to ATP hydrolysis, the enzyme also catalyzes a K^+-stimulated hydrolysis of several organic phosphates such as acetyl phosphate and *p*-nitrophenyl phosphate (Glynn & Karlish, 1975). Although it is not clear whether the *p*-nitrophenyl phosphatase (pNPPase) activity represents a partial reaction of the overall reaction mechanism or a Froehlich phosphorylation and hydrolysis reaction, it appears to represent reaction step 3 with the formation of the E_2–P intermediate followed by K^+-induced hydrolysis.

The Na^+,K^+-ATPase exhibits complex kinetics of activation with respect to ATP. During turnover conditions (Na^+,K^+,Mg^{2+}ATP), a low-affinity (0.1–2 mM) ATP regulatory site is observed, while under phosphorylation conditions (Na^+,Mg^{2+}ATP) a high-affinity (0.1–3 μM) ATP site is found. These data have generally been interpreted in terms of two independent ATP binding sites (Robinson & Flashner, 1979; Glynn & Karlish, 1976; Froehlich et al., 1970).

In addition, early immunological studies by Askari and Rao (1972) suggested the existence of two distinct active sites, one involved in ATP hydrolysis that is antigenically distinct from that component involved in the K^+-dependent hydrolysis. More recent studies [see review by Jorgensen (1982)] also suggest that the α subunit contains a phosphorylation site that is separate and distinct from the nucleotide binding site but that these two regions are in proximity due to protein folding.

In these studies, a monoclonal antibody, designated M10-P5-C11 has been characterized with respect to its effects on the Na^+, K^+, and ATP dependence of the Na^+,K^+-ATPase activity, the partial reaction steps of enzyme phosphorylation and dephosphorylation, and ATP binding. Its influence on cardiac glycoside binding has also been determined. The specificity in the antibody effects suggests that the ATP binding site is separate from the phosphorylation site and that

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an enzyme conformational change is required to put these sites in the correct arrangement for phosphorylation to occur. These studies confirm the suitability of antibodies to investigate protein structure/function relationships. In addition, a specific conformational change or reaction transition intermediate that enhances enzyme affinity for ouabain has been indentified, and we have shown that antibody binding at one site can alter the ability of another spatially distinct site to respond to normal regulatory ligand binding.

Materials and Methods

Preparation of Na^+,K^+-ATPase and Subunits. The membrane-bound enzyme was purified from the outer medulla of frozen lamb kidneys and the α and β subunits of the purified lamb Na^+,K^+-ATPase were obtained by gel filtration of the sodium dodecyl sulfate (SDS) solubilized enzyme as described by Lane et al (1979). The activity of the enzyme preparations varied from 850 to 1100 μ mol mg^{-1} h^{-1}. The enzyme and subunits were generously supplied by Dr. L. K. Lane.

Fluorescein Isothiocyanate (FITC) Labeling of Na^+,K^+-ATPase. The labeling of Na^+,K^+-ATPase with FITC (Molecular Probes, Inc.) was carried out at room temperature according to the following procedure: Na^+,K^+-ATPase (0.5 mg/mL), in 2.0 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 9.2, was incubated for 15 min in the presence of antibody M10-P5-C11 (1 mg/mL) or sheep IgG (1 mg/mL), or no additions. Then FITC [dissolved in dimethyl sulfoxide (Me_2SO)] was added to 5 μM, and the reaction was stopped after 40 min with the addition of β-mercaptoethanol to 15 μM. The mixture was centrifuged at 150000 *g* for 1 h, and the pellet was washed with 1 mM EDTA (pH 7.3), resuspended, and recentrifuged. The pellet was then resuspended in 2% SDS and 0.4 M NaOH, at half the original volume, heated for 10 min at 100 °C, and cooled, and the absorbance at 495 nm was determined. The extent of FITC labeling was quantitated with a series of FITC standards run in parallel, assuming E_{495} = 75 000 m^{-1} cm^{-1} (Carilli et al., 1982) in 50 mM Tris-HCl, pH. 7.4. The protein recovery was quantitated by the method of Lowry et al. (1951).

Preparation of F_{ab} Fragments. Purified antibody (M10-P5-C11, 2.6 mg/mL) was incubated for 2 h at 37 °C with 0.026 mg/mL papain (Sigma) in 0.1 M sodium phosphate, 0.15 M NaCl, 0.002 M EDTA, and 0.01 M cysteine, pH 7.0 (Stanworth & Turner, 1978). The reaction was stopped by the addition of *N*-ethylmaleimide to a final 0.01 M concentration. The solution was then dialyzed extensively against 0.1 M sodium phosphate, pH 8.0, and then loaded on a protein A–Sepharose column. The F_{ab} fragments eluted from the column at pH 8.0, and the F_c fragments eluted at pH 6. The completeness of the papain digestion was monitored by resolving the reaction mixture on 7.5% SDS–polyacrylamide gels in the absence or presence of 10 mM β-mercaptoethanol.

Enzyme Assays. The Na^+,K^+-ATPase activity was determined by using a coupled enzyme spectrophotometric assay (Schwartz et al., 1969) in a medium containing 30 mM histidine, 5 mM Tris–ATP (P-L Biochemicals), 10 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.36 mM NADH, 2 mM phosphoenolpyruvate, and 15 μL of pyruvate kinase/lactate dehydrogenase (Sigma, dialyzed when needed), pH 7.2. The K^+-dependent phosphatase activity (pNPPase) was measured in a medium containing 30 mM histidine, 5 mM $MgCl_2$, 4 mM *p*-nitrophenylphosphate, 5 mM KCl, and 1 mM EGTA–Tris, pH 7.4. In initial studies, the effect of the antibody on enzyme activity was determined by adding antibody to the reaction mixture in the cuvette. In most of the work reported here, however, the Na^+,K^+-ATPase was incubated with SDS (0.014–0.017%) at a 20 μg of SDS/15 μg of Na^+,K^+-ATPase ratio for 15 min at 4 °C in approximately 140 μL of Tris buffer. Antibody M10-P5-C11 or nonspecific IgG was then added to bring the solution to 180 μL and incubated with the enzyme for 15 min at 4 °C. The enzyme–antibody mixture (0.75 μg of enzyme) was then used in the assay reaction mixture for the ATPase activity determination. The ATPase reaction was initiated by the addition of varying concentrations of ATP, Na^+, or K^+ as indicated. Since the antibody and its F_{ab} fragments were purified by affinity chromatography in 0.14 M phosphate, extensive dialysis in 25 mM Tris-HCl (pH 7.4) was required to remove the phosphate. The effects of antibody in the presence of similarly dialyzed sheep IgG, or sheep IgG alone, or the dialysis buffer itself on enzyme activity and ouabain binding were routinely determined to test for nonspecific effects.

Ouabain Binding. The [^{3}H]ouabain binding was carried out at 37 °C essentially as described by Wallick and Schwartz (1974) with differing binding conditions: (a) no ligands (Tris buffer only, 50 mM, pH 7.4), (b) Mg^{2+}, (c) Mg^{2+}ATP, (d) Na^+,Mg^{2+}ATP, (e) Na^+,K^+,Mg^{2+}ATP, and (f) $Mg^{2+}P_i$. The ligand concentrations were 5 mM for the $Mg^{2+}Cl_2$, Tris–ATP, and phosphate, 100 mM for NaCl, and 1 mM for KCl. The enzyme (15 μg) and antibody M10-P5-C11 (60 μg) or sheep IgG (60 μg) were preincubated in 0.18 mL with 10 mM Tris buffer, pH 7.4, for 15 min at 0 °C. Glycoside binding, under the various ligand conditions, at 37 °C was initiated by adding [^{3}H]ouabain (1000 mCi/mmol) to obtain a final 1-mL volume and concentration of 0.1 μM. The binding experiments were carried out for various times depending upon the ligands and the particular experiment. All values are corrected for nonspecific binding as determined by the inclusion of 0.1 mM unlabeled ouabain. The enzyme was not treated with SDS because this solubilized the enzyme sufficiently to reduce enzyme binding to the 0.22-μm Millipore filters.

ATP Binding. The effect of antibody on [γ-^{32}P]ATP binding to Na^+,K^+-ATPase was determined by preincubating the Na^+,K^+-ATPase in a 180-μL mixture consisting of 15 μg of enzyme, 62 μg of immunoglobulin, 33 mM histidine, and 11 mM EDTA, pH 7.4, for 1 h at 0 °C and then adding [γ^{32}P]ATP at various concentrations to make a final 0.2-mL solution (sp act.: 100 μM ATP, 100 cpm/pmol; 2 μM ATP, 1000 cpm/pmol). After 15 min at 0 °C, the microtubes (Sarstedt) were placed in 50 Ti rotor polycarbonate centrifuge tubes containing 3 mL of water, and the tubes were centrifuged at 150000*g* for 1 h. From each reaction tube a 50-μL aliquot of the supernatant was removed and counted. The remaining supernatant was carefully removed, leaving the pellet in the tube. NCS tissue solubilizer (75 μL) was then added to each tube and allowed to incubate 30 min. The tubes were then placed in 7 mL of Budgetsolve and the cpm of [γ-^{32}P]ATP determined on a Beckman scintillation counter. Nonspecific binding was determined by the inclusion of 5 mM unlabeled ATP.

Enzyme Phosphorylation and Dephosphorylation. Steady-state phosphorylation was done at 0 °C essentially as described by Post et al. (1969). The reaction mixture (0.20 mL) contained 15 μg of Na^+,K^+-ATPase, 30 mM histidine, pH 7.4, 100 mM NaCl, 0.1–2 mM $MgCl_2$, 0–10 μg of SDS, 30–60 μg of M10-P5-C11 or control immunoglobulin, and [γ-^{32}P]ATP. In order to test the effect of the antibody on the level of phosphorylation, the buffer, antibody, and enzyme (~0.18 mL) were incubated at 0 °C for 30 min, and then the

ATP was added to initiate phosphorylation. The phosphorylation of SDS-treated enzyme was performed by mixing buffer, SDS, and enzyme (~0.13 mL) and incubating for 20 min at 0 °C, then adding antibody and incubating at 0 °C for 30 min, and then adding ATP to initiate phosphorylation. The phosphorylation reaction was terminated after 10 s at 0 °C by adding 5 mL of cold 10% trichloroacetic acid containing 0.6 mM ATP and 0.6 mM H_3PO_4, followed by filtration of the solution through 0.22-μm Millipore filters and then washing the filters 3 times with 5 mL of the stopping solution. In order to compare the relative rates of dephosphorylation and to determine the effect of antibody on the dephosphorylation step, the Na^+,K^+-ATPase was phosphorylated for 10 s, then antibody or control IgG was added, and dephosphorylation was initiated 10 s later by the addition of EDTA (10–20 mM final concentration), EDTA plus 5 mM ADP, or EDTA plus 0.1 mM KCl. Alternatively, dephosphorylation was determined by adding unlabeled ATP (0.2 mM final concentration), ATP plus 5 mM ADP, or ATP plus 0.1 mM KCl. The dephosphorylation reaction was then terminated after 5 s at 0 °C by the trichloroacetic acid, ATP, and phosphate solution.

Cell Line. The cloned hybridoma cell line secreting antibody M10-P5-C11 was generated by the fusion of mouse splenic lymphocytes from immunized mice ($CB6F_1$ from Jackson laboratory) with the SP2/0-Ag14 myeloma cell line [obtained from Shulman (1978)] according to the procedures of Galfre et al. (1977). The secreted antibody is an IgG_1 immunoglobulin with κ light chains. The monoclonality of antibody M10-P5-C11 was further suggested by isoelectric focusing of the antibody (reduced and nonreduced) on 4% polyacrylamide gel electrophoresis with an ampholine (2%) pH gradient range of 5–9.5 (O'Farrell, 1975; Cotten et al., 1973).

Purification of Antibodies. The immunoglobulins were isolated from the ascites fluid of pristane-primed mice inoculated with the cloned hybridoma cells. The ascites serum was brought to an 18% w/v sodium sulfate concentration and the immunoglobulin precipitated and collected. The IgG_1 antibodies were then purified from the serum proteins and other immunoglobulins by affinity column chromatography on a (*Staphylococcus aureus*) protein A–Sepharose (Pharmacia) column as described by Ey et al. (1978).

Determination of Antibody Binding. Antibody binding was measured by using an indirect surface adsorption binding assay similar to that developed by Engvall (1978). A β-galactosidase sheep anti-mouse IgG $F_{(ab)'_2}$ conjugate (Bethesda Research Laboratories) was utilized as previously described (Ball, 1984) to detect the antigen–antibody complex.

Other Measurements and Procedures. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. The purity of the antibody M10-P5-C11, its F_{ab} fragments, and the Na^+,K^+-ATPase and the specificity of the FITC labeling of the α subunit were confirmed by resolving the proteins on SDS–polyacrylamide gels (7.5%) prepared according to Laemmli (1970). The gels were stained with Coomassie brilliant blue. Rabbit anti-mouse IgG and IgG_1, goat anti-mouse IgG [$F_{(ab)'_2}$ fragment specific], and goat anti-mouse IgG (F_c fragment specific) immunoglobulins were employed with the double-diffusion method of Ouchterlony (1959) to identify the isolated F_{ab} fragments.

RESULTS

Determination of Antibody Binding Specificity and Affinity. The hybridoma cell line producing antibody M10-P5-C11 reported here was identified by screening the hybridoma cell culture media of mouse spleen–mouse myeloma hybrids for the presence of anti-holoenzyme antibodies. An indirect solid surface adsorption assay was used for the screening process, and the cell line was isolated by dilution cloning. Ouchterlony double-diffusion precipitation using IgG subclass specific anti-mouse immunoglobulins demonstrated M10-P5-C11 to be a mouse IgG_1 (κ light chain) immunoglobulin. Titer or dilution binding curves using affinity column purified antibody showed that half-maximal binding to Na^+,K^+-ATPase occurred at approximately 7 nM, while the titer value toward isolated α-subunit was 1500 nM. No antibody binding to the β subunit of the lamb enzyme was detected, nor was any binding to purified rat kidney Na^+,K^+-ATPase observed. The antibody did not bind to electrophoretic or Western blots of the purified enzyme. By these criteria, the antibody was largely specific for the "native" lamb enzyme. Competition binding studies also showed the epitope for antibody M10-P5-C11 to be distinct from that of antibody M7-PB-E9, which has been characterized previously (Ball, 1984).

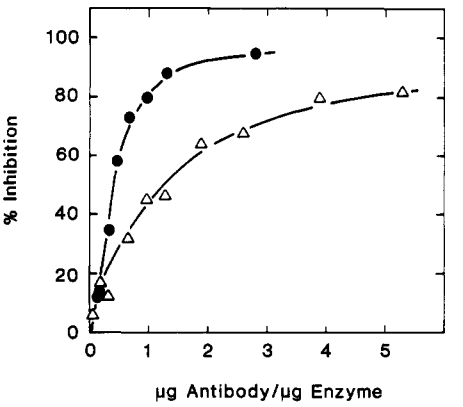

FIGURE 1: Effect of antibody on ATPase activity of lamb kidney Na^+,K^+-ATPase. The closed circles (●) represent extent of inhibition of enzyme activity with increasing concentrations of antibody M10-P5-C11; the open triangles (△) represent effect of F_{ab} fragments. The SDS-treated enzyme was preincubated with antibody for 15 min as described under Materials and Methods before addition to reaction assay mixtures. Each point represents the average of two to three determinations.

Effect of Antibody on ATPase Activity. Antibody M10-P5-C11 was found to maximally inhibit about 70% of the Na^+,K^+-ATPase activity. However, since the enzyme is purified in a particulate, vesicular form with the enzyme presumably oriented randomly to both sides of the membrane, the incomplete inhibition could result from the inaccessibility of some antigenic sites. Therefore, a partial "solubilization" of the enzyme was attempted with SDS. The addition of SDS to a 1.3/1.0 weight ratio of SDS/enzyme gave a 20–30% increase in the ATPase activity. This increase in activity was accompanied by an increase in the apparent enzyme affinity for ATP, and the extent of antibody inhibition was increased to greater than 90%. Figure 1 shows the effect of antibody concentration upon activity of the SDS-treated Na^+,K^+-ATPase. Fifty percent inhibition was achieved at about 2 nM antibody, with an antibody to enzyme stoichiometry of slightly less than 1:1.

In the experiment shown in Figure 1, antibody and enzyme were incubated in the absence of binding ligands before enzyme activity was assayed. Similar inhibition was obtained when the antibody was added directly to the assay mix in the absence of ATP or when the antibody was added during ATP hydrolysis. Antibody binding and resultant inhibition were not sensitive to different ligand conditions. These results are in marked contrast to those obtained by Schenk et al. (1984)

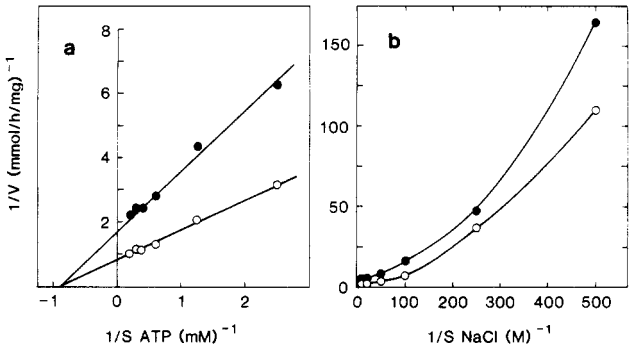

FIGURE 2: Effect of antibody M10-P5-C11 on ATP and NaCl concentration dependence of Na^+,K^+-ATPase activity. (a) Open circles (○) represent the enzyme activity in the absence of antibody and closed circles (●) the activity in the presence of added antibody. The antibody and SDS-treated enzyme were preincubated for 15 min before initiation of reaction with ATP. Each reaction cuvette contained approximately 0.75 μg of enzyme in 1.25 mL and sufficient antibody for about 50% inhibition. The data are plotted as a Lineweaver–Burk plot. (b) Open circles (○) represent enzyme activity in absence of and closed circles (●) the activity in the presence of added antibody. The reaction is initiated with NaCl. Each point represents the average of two to three determinations. Hill plots of data gave $n_H = 1.1$ for ATP, 1.3 for KCl, and 1.7 for NaCl. Antibody had no effect on Hill coefficients.

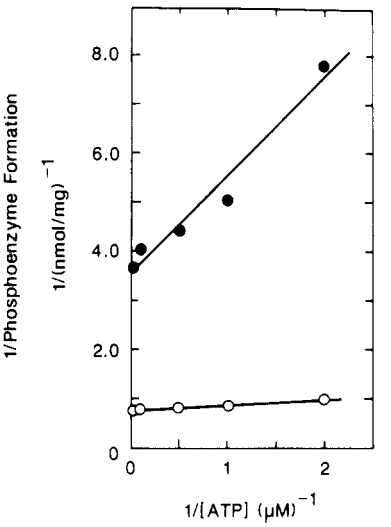

FIGURE 3: Effect of antibody M10-P5-C11 on ATP dependence of Na^+,K^+-ATPase phosphoenzyme intermediate formation. Open circles (○) and closed circles (●) represent phosphoenzyme formation in absence and presence of antibody, respectively. Enzyme (15 μg) was treated with SDS, then antibody added, and the [γ-^{32}P]ATP phosphorylation carried out for 10 s at 0 °C as described under Materials and Methods. Each point was done in triplicate.

using a monoclonal antibody (9-A5) directed against the rat kidney enzyme. Their antibody (9-A5) blocks the ATPase activity, but they report that it does not bind in the absence of a cation (Na^+ or K^+) and ATP prevents antibody binding.

Effect of F_{ab} Fragments on ATPase Activity. Antibody M10-P5-C11 was also subjected to papain digestion, the F_{ab} fragments were isolated, and their effect on enzyme activity was determined (Figure 1).

Although higher concentrations were required than with the intact immunoglobulin, the F_{ab} fragments of the antibody also inhibited the ATPase activity. Determination of the competition between F_{ab} fragments and intact antibody for binding to the holoenzyme also demonstrated that the F_{ab} fragments bind less tightly than the antibody. This reduced affinity of F_{ab} fragments relative to that of the intact immunoglobulin is consistent with previous reports (Petrossian & Owicki, 1984; Mason & Williams, 1980).

Effect of Antibody on ATP, Na^+, and K^+ Dependency of ATPase Activity. The effect of varying the ATP concentration on the extent of antibody inhibition was determined at an antibody concentration that inhibited about 50% of the ATPase activity at a 5 mM ATP concentration. The data, presented as a Lineweaver–Burk plot (Figure 2a), suggest that antibody inhibition is noncompetitive with respect to ATP. Similarly, a noncompetitive inhibition or mixed inhibition was observed when either the Na^+ (Figure 2b) or K^+ (not shown) concentration was varied. The extent of antibody inhibition showed little variation over the range of substrate concentrations used. The cooperativity of the nonequivalent Na^+ sites and K^+ sites, as illustrated by the nonlinear inverse plot, was not altered by the presence of antibody.

Determination of Antibody Effect on Partial Reactions of ATPase Activity. The reaction mechanism for the ATPase activity involves at least a four-step process, with the formation of the Na^+-dependent $E_1\sim P$ intermediate, its conversion to the E_2–P intermediate, the K^+-dependent dephosphorylation, and the $K^+\cdot E_2 \rightarrow Na^+\cdot E_1$ conversion. In these studies, the effect of antibody on the steady-state level of the Na^+,-Mg^{2+}ATP-dependent phosphoenzyme intermediate formation at 0 °C was determined. The antibody caused about a 50% inhibition of phosphoenzyme formation, and half-maximal inhibition was achieved at a slightly less than 1:2 molar ratio of antibody to αβ monomer. As with the ATPase activity, SDS treatment of the enzyme (10 μg of SDS/15 μg of NKA) increased enzyme affinity for ATP (with the apparent K_m value of ATP shifted from about 0.3 to 0.10 μM) and increased the extent of antibody inhibition to 80–90%. At subsaturating ATP levels, a 20–40% increase in the level of phosphoenzyme was observed. Figure 3 shows the Lineweaver-Burk plot of the effect of varying the ATP concentration on phosphoenzyme formation. The antibody acts as a mixed-type inhibitor in reducing the level of phosphorylation.

Studies of phosphoenzyme dephosphorylation were then done to determine possible antibody effects on the hydrolysis steps and $E_1\sim P \rightleftarrows E_2$–P conversion. In initial studies, the enzyme was phosphorylated for 10 s, and then dephosphorylation conditions were initiated and terminated after 5 s. When the K^+-dependent and the ADP-sensitive ($E_1\sim P + ADP \rightleftarrows E_{Na} + ATP$) dephosphorylation steps were monitored, no significant differences between the rates of dephosphorylation with added ATP or with ATP plus ADP were observed, and no distinction between the $E_1\sim P$ and E_2–P intermediate forms was possible. Early studies by Post et al. (1969) and Fahn et al. (1966), especially those using the *N*-ethylmaleimide-(NEM-) modified enzyme, suggested that the $E_1\sim P \rightleftarrows E_2$–P conversion was Mg^{2+}-dependent and that $E_1\sim P$ formation was favored at low Mg^{2+} concentrations. Therefore, both phosphorylation and dephosphorylation conditions were varied in order to identify the ADP-sensitive dephosphorylation. The dephosporylation reaction was observed subsequent to phosphorylation under 2 mM Mg^{2+} (Table IA) and 0.1 mM Mg^{2+} (Table IB) conditions with the addition of either (A) 0.1 mM unlabeled ATP to dilute the [γ-^{32}P]ATP but with phosphorylation continuing or (B) EDTA (10 mM) to chelate the 0.1 mM Mg^{2+} and prevent further phosphorlyation. The extent of phosphorylation was, however, the same at either 2 mM or 0.1 mM Mg^{2+}. In addition, while K^+ clearly increased the rate of dephosphorylation over that of the EDTA or ATP added controls, no significant difference between the dephosphorylation controls and controls plus ADP was observed. These studies confirm the work of Klodes and Skou (1975) and Kanazawa et al. (1970), which suggested phosphoenzyme

Table I: Effect of Mg^{2+} Concentration on Phosphoenzyme Formation and Dephosphorylation[a]

	reaction conditions	phosphoenzyme formation
(A) 2 mM Mg^{2+}	phosphorylation conditions (10 s)	841 pmol/mg of protein (100%)
	dephosphorylation (5 s) additions	
	0.2 mM ATP	51%
	ATP + 5 mM ADP	52%
	ATP + 0.1 mM KCl	5%
(B) 0.1 mM Mg^{2+}	phosphorylation conditions (10 s)	899 pmol/mg of protein (100%)
	dephosphorylation (5 s) additions	
	EDTA (10 mM)	48%
	EDTA + 5 mM ADP	55%
	EDTA + 0.1 mM KCl	5%

[a] Enzyme (15 μg) was phosphorylated for 10 s at 0 °C in 30 mM histidine, pH 7.4. The dephosphorylation reactions were terminated after 5 s at 0 °C. Phosphorylation conditions were (A) 2 mM Mg^{2+}, 100 mM Na^+, and 2 μM [γ-^{32}P]ATP and (B) 100 μM Mg^{2+}, 100 mM Na^+, and 2 μM [γ-^{32}P]ATP. Dephosphorylation conditions are given in final concentrations of additions. Values are given as percentages relative to initial phosphorylation levels. All determinations were done in triplicate. Preincubation of enzyme with antibody M10-P5-C11 or sheep IgG before enzyme phosphorylation had no significant effect on relative dephosphorylation rates.

interconversion is not Mg^{2+} dependent and that under these conditions the E_2–P intermediate form predominates. However, when the phosphorylation was done with 1 M Na^+ as reported by Yoda and Yoda (1982), ADP-sensitive dephosphorylation was observed (Table IIB).

To clarify the possible effects that M10-P5-C11 might have on the dephosphorylation steps as distinct from the phosphorylation steps, the following protocol was devised: the enzyme was phosphorylated for 10 s at 0 °C with 20 μM [γ-^{32}P]ATP. Then, antibody was added, and 10 s later the dephosphorylation conditions were initiated. Dephosphorylation was allowed to proceed for 5 s. This procedure avoided the possibility that the measured phosphorylation and dephosphorylation were occuring only with antibody-free enzyme. Our studies showed that approximately 85% of maximal inhibition of phosphoenzyme formation was achieved within 10 s after addition of antibody. When the antibody was added after phosphorylation, it blocked rephosphorylation of the enzyme, but initially (5–15 s) it had less effect on the level of phosphoenzyme intermediate than adding excess EDTA or cold ATP. Table II shows that under both 1 and 0.1 M Na^+ conditions the antibody had little or no effect on either the ADP- or K^+-stimulated dephosphorylation. The antibody did not inhibit E_2–P hydrolysis, $E_1 \sim P \rightarrow E_2$–P conversion, or the $E_1 \sim P \rightarrow E_{Na}$ + ATP reversal.

The K^+-dependent hydrolysis of *p*-nitrophenyl phosphate is also considered to be a measure of the K^+-catalyzed dephosphorylation. Antibody had no effect on the pNPPase activity. This provided additional evidence that the antibody specifically inhibited phosphorylation by ATP but not the subsequent reaction steps.

Antibody Effects on Ouabain Binding. The Na^+,K^+-ATPase also functions as the receptor for cardiac glycosides. The enzyme affinity for the glycosides is highly dependent upon the ligands present during glycoside binding. In the absence of any ligands, little binding occurs, while an increasing rate of binding is observed with the following ligand conditions: (1) Mg^{2+}; (2) Mg^{2+}ATP; (3) K^+,Na^+,Mg^{2+}ATP; (4) Na^+-Mg^{2+}ATP; (5) $Mg^{2+}P_i$. As shown in Table III, equilibrium binding studies done with 0.1 μM [^{3}H]ouabain indicated that antibody M10-P5-C11 altered binding under Mg^{2+}ATP and K^+,Na^+Mg^{2+}ATP conditions, but did not affect [^{3}H]ouabain binding in the presence of Mg^{2+} or MgP_i. Determination of the rates of binding confirmed a ligand-specific antibody effect on ouabain binding (Table III). The antibody did not alter the rate of ouabain binding with Mg^{2+} and $Mg^{2+}P_i$ but under Mg^{2+}ATP conditions reduced the rate by 60%, and it essentially prevented the ATP-induced stimulation of the [^{3}H]-ouabain binding rate. It also reduced the rate of binding, or stimulation by ATP, under Na^+,Mg^{2+}ATP and K^+,Na^+,-Mg^{2+}ATP conditions. This effect is specific for M10-P5-C11 binding. Previous studies (Ball, 1984) identified an antibody (M7-PB-E9) that simulates ATP in enhancing the rate of ouabain binding in the presence of Mg^{2+}, while nonspecific IgG and three other Na^+,K^+-ATPase-specific monoclonal antibodies were found to have no effect on [^{3}H]ouabain binding. These data as well as the inhibitory effects on enzyme activity and phosphorylation suggested that M10-P5-C11 might block the binding of ATP to enzyme. The antibody did not, however, appear to alter Mg^{2+},Na^+, or K^+ effects on enzyme activity or ouabain binding.

Table II: Effect of Antibody M10-P5-C11 on Phosphoenzyme Dephosphorylation[a]

	reaction conditions	percent phosphorylation: control (sheep IgG)	percent phosphorylation: antibody (M10-P5-C11)
(A) 0.1 M Na^+	phosphorylation conditions (10 s, 1425 pmol/mg of protein)		
	additions		
	antibody (10 s)	100 (1450 pmol/mg)	100 (1038 pmol/mg)
	dephosphorylation (5 s)		
	EDTA (10 mM)	48	50
	EDTA + 5 mM ADP	57	60
	EDTA + 0.1 mM KCl	3	4
(B) 1 M Na^+	phosphorylation conditions (10 s, 975 pmol/mg of protein)		
	additions		
	antibody (10 s)	100 (1164 pmol/mg)	100 (870 pmol/mg)
	dephosphorylation (5 s)		
	EDTA (20 mM)	45	42
	EDTA + 5 mM ADP	3	10
	EDTA + 0.1 mM KCl	33	33

[a] Na^+,K^+-ATPase (15 μg) was phosphorylated for 10 s at 0 °C in histidine buffer, pH 7.4, containing (A) 0.1 M Na^+, 0.1 mM Mg^{2+}, and 20 μM [γ-^{32}P]ATP and (B) 1 M Na^+, 2 mM Mg^{2+}, and 20 μM [γ-^{32}P]ATP. Excess antibody M10-P5-C11 (60 μg) or sheep IgG (60 μg) was added, and 10 s later dephosphorylation conditions were initiated. Dephosphorylation conditions (5 s, 0 °C) are given as final concentrations after addition. Dephosphorylation values are given as percentages relative to phosphorylation levels after addition of antibody. All determinations were done in triplicate. The average error less than 5%.

Table III: Effect of Antibody M10-P5-C11 on [^{3}H]Ouabain Binding[a]

	relative percent [^{3}H]ouabain bound, ligands present				
	Mg^{2+}	MgATP	Na,MgATP	K,Na,MgATP	MgP_i
equilibrium binding					
control	100	100	100	100	100
plus sheep IgG	100	101	104	109	95
plus antibody M10-P5-C11	93	53	102	74	100
relative rates of binding					
control	100	100	100	100	100
plus sheep IgG	100	97	109	103	99
plus antibody M10-P5-C11	98	40	85	50	97

[a] Determination of equilibrium binding: The Na^+,K^+-ATPase (5 or 15 μg) was preincubated with an excess of antibody M10-P5-C11 (15 or 60 μg), nonspecific sheep IgG, or no immunoglobulins for 15 min at 0 °C. The specified ligands and [^{3}H]ouabain (to give 0.1 μM) were added at 37 °C. Ligand concentrations are given under Materials and Methods, and equilibrium binding times were as follows: Mg^{2+} and MgATP, 4–6 h; Na^+,Mg^{2+}ATP and K^+,Na^+,Mg^{2+}ATP, 2 h; MgP_i, 30 min to 1 h. Equilibrium [^{3}H]ouabain binding was 2138 pmol/mg of protein with MgP_i and 1303 pmol of [^{3}H]ouabain/mg of protein in the presence of Mg^{2+}. Values are given as percent relative to the control for each binding condition. Determination of relative rates of binding: The rate of [^{3}H]ouabain binding at 37 °C under various ligands conditions was determined subsequent to the preincubation of Na^+,K^+-ATPase with antibody M10-P5-C11 or sheep IgG. (a) Binding times in the presence of Mg^{2+} and Mg^{2+}ATP were for 10 or 15 min; (b) Na^+,Mg^{2+}ATP and K^+Na^+,Mg^{2+}ATP rates are for 10 min; (c) the [^{3}H]ouabain binding for $Mg^{2+}P_i$ conditions was determined at 5 min. The data are presented relative to the rate of binding for each ligand condition with no antibody present. The [^{3}H]ouabain binding rates were 21 and 218 pmol min^{-1} (mg of protein)$^{-1}$ for Mg^{2+} and $Mg^{2+}P_i$ conditions, respectively. Values are averages of at least two experiments each done in triplicate.

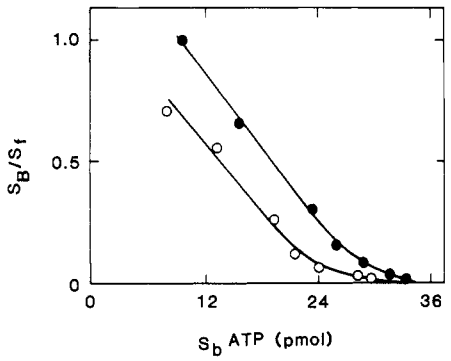

FIGURE 4: Effect of antibody on [γ-^{32}P]ATP binding to Na^+,K^+-ATPase. Open circles (○) represent ATP binding (10–0.1 μM) to Na^+,K^+-ATPase (15 μg) in the presence of sheep nonspecific IgG (62 μg) and closed circles (●) in the presence of antibody M10-P5-C11 (62 μg). The procedure was as described under Materials and Methods. The data were fit by a ligand binding program developed by Munson and Rodbard (1980) and converted for use with an Apple IIe computer by Dr. Martin Teicher, Department of Psychiatry, Harvard Medical School. The two-parameter fit gave an F ratio test value of 25.7 or a $P < 0.05$. The F ratio = $(SS_1 - SS_2/(df_1 - df_2)/(SS_2/df_2)$ and is based on the "extra sum of squares" principle (Draper & Smith, 1966). Each point was done in triplicate.

Antibody Effect on ATP Binding. The effect of M10-P5-C11 on ATP binding was determined by preincubating the enzyme and antibody together for 1 h at 0 °C and then adding [γ-^{32}P]ATP for 15 min at 0 °C. The enzyme was pelleted by centrifugation, and the enzyme-bound [γ-^{32}P]ATP in the pellet and the free [γ-^{32}P]ATP in the supernatant were determined. Figure 4 shows the Scatchard plot analysis of ATP binding in the presence of nonspecific sheep IgG and that with antibody M10-P5-C11. There was a higher rather than lower level of ATP binding when M10-P5-C11 was present, and this might be explained by an increased recovery of the pelleted enzyme with bound antibody. Also as reported previously by Koepsell (1978) for ATP binding to the rat kidney Na^+,K^+-ATPase, these data indicated the presence of "high"- and "low"-affinity ATP sites. The dissociation constants for the high-affinity sites, 0.09 μM with M10-P5-C11 present and 0.10 μM with sheep IgG, and the extrapolated total ATP binding (2.4 nmol/mg of enzyme) were nearly identical. The low-affinity ATP sites had K_d values of approximately 6 μM. The monoclonal antibody caused no decrease in ATP binding to the enzyme.

Antibody Effect on Fluorescein Isothiocyanate Labeling of Enzyme. Several studies have shown that fluorescein 5′-isothiocyanate (FITC) specifically inactivates the Na^+,K^+-ATPase and prevents ATP binding by covalently labeling a single site on the α subunit of the enzyme (Karlish et al., 1979; Farley et al., 1984; Kirley et al., 1984). The labeling and the inactivation of enzyme activity by FITC are prevented by ATP. Therefore, it is presumed that FITC reacts at or near the ATP binding site of the enzyme. The similar inhibitory effects of antibody and FITC upon enzyme phosphorylation but not dephosphorylation suggested the possibility that they might bind at the same or overlapping regions of the α subunit. Therefore, the effect of antibody on FITC labeling was determined. Initial studies demonstrated that the presence of nonspecific immunoglobulins (IgG, 1–2 mg/mL) only slightly reduced the rate at which 5 μM FITC inactivated the enzyme (0.5 mg/mL) and did not affect the extent of inactivation. After 40 min, the enzyme was more than 90% inactivated (Table IV). Gel electrophoresis (Figure 5) of the reaction samples showed that the α subunit of Na^+,K^+-ATPase was labeled and neither nonspecific sheep IgG nor M10-P5-C11 was labeled with FITC. Finally, under the FITC labeling conditions, a 2:1 stoichiometry of antibody to enzyme was found to inhibit approximately 92% of the enzyme activity. Quantitation of FITC labeling then showed that the extent of labeling of the α subunit was not affected by either nonspecific IgG or by antibody (Table IV). In addition, the stoichiometry of labeling of approximately 2.6 nmol/mg of protein is essentially identical with that reported by Kirley et al. (1984) using a similar preparation of the lamb kidney enzyme.

Table IV: Effect of Antibody M10-P5-C11 on FITC Labeling of Na^+,K^+-ATPase[a]

sample	% ATPase activity with addition of		quantitation of FITC labeling (nmol/mg)
	none	FITC	
Na^+,K^+-ATPase	100	8	2.6
Na^+,K^+-ATPase plus sheep IgG	100	8	2.9
Na^+,K^+-ATPase plus M10-P5-C11	6		2.7

[a] The FITC labeling and quantitation was performed as described under Materials and Methods, with data given as the averages of two experiments each done in duplicate.

Discussion

We have obtained several monoclonal antibodies directed

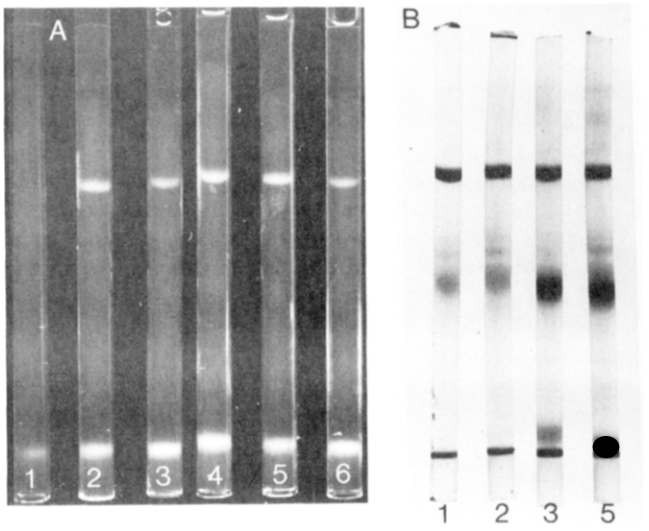

FIGURE 5: Polyacrylamide gel electrophoresis demonstrating specificity of FITC labeling of α subunit of Na^+,K^+-ATPase in the presence of immunoglobulin. (A) The fluorescence of FITC as seen by UV illumination. Gels were as follows: (1) Na^+,K^+-ATPase, no FITC; (2) Na^+,K^+-ATPase, plus FITC; (3 and 4) Na^+,K^+-ATPase, FITC, and sheep IgG (1:1 and 2:1 ratio of IgG to enzyme); (5 and 6) Na^+,K^+-ATPase, FITC, and M10-P5-C11 (1:1 and 2:1 ratio of antibody to enzyme). All gels contained 10 μg of Na^+,K^+-ATPase applied in sample buffer containing SDS, urea, and β-mercaptoethanol. (B) Protein resolution of Na^+,K^+-ATPase labeled by FITC in the presence of immunoglobulin. The gels were stained with Coomassie blue after photographing the UV fluorescence. The gels are 1, 2, 3, and 5 as shown in (A).

against the α subunit of the lamb kidney Na^+,K^+-ATPase (Ball et al., 1982). These antibodies were raised to the holoenzyme, but they have a higher affinity toward the isolated, denatured α subunit than the holoenzyme. Antibody M10-P5-C11 is unique in its high affinity for the active native lamb kidney enzyme and low affinity toward the isolated α subunit. In addition, M10-P5-C11 is an effective inhibitor of the ATPase activity. Antibody M10-P5-C11 clearly inhibits phosphorylation of the enzyme without altering the dephosphorylation steps. It acts as a noncompetitive inhibitor, reducing the V_{max} of the reaction without any apparent alteration of enzyme affinity for ATP or the interactions between the nonequivalent Na^+ or K^+ binding sites. Since antibody binding under the assay conditions should be essentially irreversible, the antibody could alter ATP binding, but because it also removes active enzyme from the system, it does not alter the kinetics of unbound enzyme. However, the antibody does not affect ATP binding or FITC reactivity. In addition, binding appears to be independent of any specific ligand conditions. These results are in contrast to those obtained previously in this laboratory with M7-PB-E9 and with the work of Schenk et al. (1984). The antibody M7-PB-E9 acted as a partial competitive inhibitor with respect to ATP and could substitute for ATP in stimulating ouabain binding. Schenk et al. (1984) have isolated an antibody, 9-A5, that blocks the rat kidney enzyme's ATPase activity but that requires Na^+ or K^+ for binding. It can also be prevented from binding by the presence of ATP. Thus, antibody 9-A5 appears to block ATP binding by being directed against or in the proximity of the ATP binding site. Antibody M10-P5-C11 inhibits through a different mechanism; it binds at an antigenic site distinct from either the ATP or phosphorylation site and appears capable of recognizing this site as the enzyme undergoes the normal conformational alterations occurring during catalytic turnover.

The data support the concept that the observed inhibition results from the restriction or blocking of a conformational transition of the enzyme. Antibody binding appears to prevent the polypeptide region of the ATP binding site from repositioning the ATP molecule into the correct orientation for the transfer of the γ-phosphoryl from ATP to the protein's phosphorylation site. M10-P5-C11 inhibits this step without altering either ATP binding, phosphoenzyme intermediate interconversion, or the reaction steps of the dephosphorylation reaction. This could be achieved by "freezing" a small region of the active site or a steric blocking by the antibody that effects phosphorylation. The inhibition does not appear to be due to nonspecific steric hindrance because the F_{ab} fragments, one-third the molecular weight of the antibody, also inhibit. The specificity of the antibody toward the native enzyme suggests that it binds a discontinuous antigenic determinant. Binding to two disparate regions of the polypeptide chain could prevent a specific conformational change.

These studies have demonstrated an uncoupling of Mg^{2+}-ATP binding from an ATP-caused increase in enzyme affinity for ouabain. It appears that a unique step or conformational change occurs after Mg^{2+}ATP binding but before phosphoenzyme formation. This conformational change or intermediate step that enhances ouabain binding has been suggested but not demonstrated by previous fluorescence labeling work (Taniguchi et al., 1982, 1984). Recent kinetic studies by Petithory and Jencks (1986; personal communication) have identified an analogous conformational change for the Ca^{2+}-ATPase of sarcoplasmic reticulum.

In the presence of Mg^{2+}, ATP binding causes a conformational alteration that is propagated or transduced by the polypeptide chain through the plasma membrane from the intracellular ATP binding region to the extracellular cardiac glycoside receptor. Phosphorylation of the enzyme is not required for this effect. This has been demonstrated by the observations that Mg^{2+}ATP and Mg^{2+} plus antibody M7-PB-E9 and not just MgP_i effect a stimulation in the rate of ouabain binding (Ball, 1984).

It is not clear to what extent Na^+ regulates the proposed ATP repositioning step, but it is clear that Mg^{2+} and ATP are required for the conformational change that stimulates the rate of ouabain binding. Na^+ reduces the effectiveness of the antibody inhibition while it additionally increases the enzyme's affinity for ouabain and is essential for phosphoenzyme formation. Therefore, Na^+ may reduce the overall distance between the ATP binding and phosphorylation sites and then be directly involved in the formation of the transition state of the phosphorylation reaction. This distinction between two different Na^+ effects agrees with the proposal of Scarborough (1982) of two identical Na^+ sites, which effect a conformational change that leads to a first transition state and a third nonidentical Na^+ binding site that enables Na^+ to directly promote the phosphorylation reaction. The addition of K^+ reduces enzyme affinity for ATP (Moczydlowski & Fortes, 1981a,b) and ouabain, and it partially restores the antibody's effectiveness in inhibiting the ATP-induced stimulation of ouabain binding.

A likely structural model is that the active site is composed of two separate sequence regions and the antibody's epitope is an exposed intracellular region within a groove or cleft of the active site region. The catalytic reaction appears to involve the binding of ATP to the His-Leu-Leu-Val-Met-Lys-Gly-Ala-Pro-Glu-Arg (Farley et al., 1984) region of the α subunit. This region is in proximity to the aspartic acid of the phosphorylation site (Bastide et al., 1973) as the result of the folding pattern of the α subunit peptide chain. After Mg^{2+}-ATP binding, a conformational change occurs that alters

ouabain binding and places the ATP in closer proximity to the phosphorylation site. The presence of Na^+ allows for the repositioning and then the phosphorylation steps. M10-P5-C11 blocks the first step and inhibits phosphorylation. The distinct nature of these two components of the active site region has been suggested earlier by immunological (Askari, 1974), chemical modification (Henderson & Askari, 1976), NMR, and structural studies [see Jorgensen (1982)].

Therefore, the concept that the active site is composed of two distinct, somewhat independent regions of the α subunit is supported by these studies of enzyme function as well as chemical labeling and sequence studies. This complexity in the active site has an important consequence in that it increases the avenues for regulation of the enzyme. For example, ouabain binding and the subsequent inhibition of activity are regulated by ligand binding at both or either the ATP site or the phosphorylation site.

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Registry No. MgATP, 1476-84-2; ATPase, 9000-83-3; ouabain, 630-60-4.

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