Immunochemical Characterization of a Functional Site of (Na^+,K^+)-ATPase†

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ABSTRACT: Several hybridoma cell lines secreting antibodies specific to the membrane (Na^+,K^+)-dependent ATPase from lamb kidney medulla have been isolated by using the methods developed by Kohler and Milstein. One of these antibodies (designated M7-PB-E9) has been shown to be directed against a functional epitope or antigenic site of the catalytic (α) subunit of the enzyme. Although this antibody was raised to the "native" holoenzyme, it has a higher apparent affinity toward the isolated, delipidated, and inactive α subunit than toward the holoenzyme. This antibody shows a 10-fold faster initial rate of binding to the α subunit than to the holoenzyme. The antibody dissociation rates from both isolated α subunit and holoenzyme are similarly slow, and the binding can be considered a pseudoirreversible reaction. By binding at this site, the antibody, however, acts like a "partial competitive inhibitor" with respect to ATP and acts as an uncompetitive or mixed competitive inhibitor with respect to the Na^+ and K^+ dependence of ATPase hydrolysis. This antibody also does not alter the cooperativity at either the Na^+ or the K^+ sites. The antibody causes a partial inhibition of the Na^+- and MgATP-dependent phosphoenzyme intermediate formation but has no effect on either ADP $\rightleftharpoons$ ATP exchange or the K^+-stimulated dephosphorylation step. In addition, the K^+-dependent *p*-nitrophenylphosphatase activity of the enzyme was not affected. In the presence of Mg^{2+}, the antibody stimulates the rate of cardiac glycoside binding ([^{3}H]ouabain) to the (Na^+,K^+)-ATPase. The data demonstrate the sensitivity of antibody binding to some alterations in the structure and/or the environment of the antibody binding site region of the α subunit. The data suggest that the antibody is directed against an epitope of the enzyme such that it can compete with ATP and can induce an "ATP-like" conformational change in the enzyme that leads to an increase in the enzyme affinity for ouabain.

Antibodies are finding increasing use as tools to study the structure and function of membrane molecules [see reviews by Oppenheim et al. (1977) and Williams (1977)]. Recently, the application of immunological techniques has made it possible to obtain new information concerning the antigenic properties of the membrane-bound (Na^+,K^+)-ATPase (Ball & Schwartz, 1982; Schellenberg et al., 1981; Girardet et al., 1981; McDonough et al., 1982). With the ability to produce single-site-specific or monoclonal antibodies, it is now possible to identify specific regions of (Na^+,K^+)-ATPase, to determine the ability of antibodies to cause site-specific alterations in enzyme function, and to study the mechanisms of these effects.

The (Na^+,K^+)-dependent ATPase is of considerable interest because it actively regulates the transport of Na^+ and K^+ across the cell membrane, and it is the pharmacological receptor for cardiac glycosides. This enzyme displays a complex array of interactions with Na^+, K^+, Mg^{2+}, P_i, and ATP which regulate its (Na^+,K^+)-ATPase activity and cardiac glycoside binding [see reviews by Glynn & Karlish (1975), Wallick et al. (1979), Robinson & Flashner (1979), and Jorgensen (1982)]. The (Na^+,K^+)-ATPase is composed of two major peptides, the catalytic (α) subunit ($M_r \simeq 100\,000$) and the glycoprotein (β) subunit ($M_r \simeq 50\,000$), and perhaps a low molecular weight protein (γ) ($M_r \simeq 12\,000$) (Forbush et al., 1978; Collins et al., 1982). Both the digitalis "receptor" and the ATP binding site appear to reside mainly on the α subunit. No clear, functional role for the β subunit or the putative γ component has been identified.

Several investigators have raised polyclonal rabbit antibodies against various preparations of the enzyme and its isolated subunits (Averdunk et al., 1969; Askari, 1974; Rhee & Hokin, 1974; Jean & Albers, 1977; Koepsell, 1978, 1979; Kyte, 1974; Michael et al., 1977). However, because of the heterogeneous nature of these antibodies, the results of many of these studies have been confusing and, in some cases, contradictory.

In this laboratory, several hybridoma cell lines produced by fusing SP2/0-Ag14 mouse myeloma and mouse spleen cells have recently been isolated (Ball et al., 1982). In this study, one of these antibodies, designated M7-PB-E9, which has been shown to partially inhibit the (Na^+,K^+)-ATPase activity, has been characterized with respect to its effects on the Na^+, K^+, and ATP dependence of the (Na^+,K^+)-ATPase and the K^+-dependent *p*-nitrophenylphosphatase activities. The effects of this antibody on the partial reaction steps of enzyme phosphorylation and dephosphorylation and on ouabain binding under several different binding conditions were investigated. This antibody can distinguish "native" and "nonnative" conformations of the catalytic subunit. This antibody also appears to bind at a functionally distinct portion of the catalytic subunit and to induce an "ATP-like" conformational change in the enzyme.

Materials and Methods

Cell Line. The anti-α subunit antibody-secreting cell line M7-PB-E9 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 according to the procedures of Galfre et al. (1977). The secreted antibody has been shown to be an IgG_1 immunoglobulin with κ light chains (Ball et al., 1982).

The monoclonality of antibody M7-PB-E9 was further suggested by isoelectric focusing of cell-culture supernatant IgG fractions. Isoelectric focusing of reduced and nonreduced antibodies was done by using 4% polyacrylamide disc gel electrophoresis with an ampholine (2%) pH gradient range of 5–9.5 (Cotton et al., 1973; O'Farrell, 1975).

Purification of Antibodies. The immunoglobulins were isolated from the ascites fluid of pristane-primed mice inoc-

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ulated with the cloned hybridoma cells. The collected fluid was subjected to 18% sodium sulfate precipitation, and the IgG fractions were collected. The IgG_1 antibodies were then obtained 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 or an enzyme-linked immunosorbent assay (ELISA) method similar to that developed by Engvall (1978). Microtiter plate wells (Cooke flexible 96-well plates) were first treated for 30 min with 100 μL/well test proteins or antigen at 0.1–0.75 mg/mL as detailed in each particular experiment. The antigen-containing solution was removed, and wells were washed with buffer A [5 mg/mL bovine serum albumin (BSA)–10 mM tris(hydroxymethyl)aminomethane (Tris)–150 mM NaCl, pH 7.4], then exposed to the antibody diluted in buffer A for various times as detailed, and washed again with buffer B (5 mg/mL BSA, 1.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.05% Triton X-100, and 0.05% sodium azide), and then a β-galactosidase–sheep anti-mouse IgG $F(ab)'_2$ conjugate (Bethesda Research Laboratories) in buffer B was added for 90 min. The bound second antibody was detected by using *o*-nitrophenyl β-galactoside as substrate for the β-galactosidase conjugate. After a 10-min incubation, the reaction was terminated with 0.5 M Na_2CO_3 and each sample transferred to a fresh well, and the absorbancy of the reaction solutions was determined at 405 nm.

Preparation of (Na^+,K^+)-ATPase and Subunits. The membrane-bound (Na^+,K^+)-ATPase was purified from the outer medulla of frozen lamb kidneys according to the procedure of Lane et al. (1979). The α subunit of the purified lamb (Na^+,K^+)-ATPase was obtained by gel filtration of the sodium dodecyl sulfate (SDS)-solubilized holoenzyme by procedures that have been described by Lane et al. (1979) and by Reeves et al. (1980). The enzyme and α subunit were generously supplied by Dr. L. K. Lane. SDS was removed from the proteins by chromatography on AG-1-X2 resin in a buffer containing 6 M urea, 0.05 M NaH_2PO_4, 1 mM β-mercaptoethanol, and 0.01% NaN_3, pH 8.0. The protein samples were then dialyzed extensively against the buffer without urea.

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, 2.5 mM Na_2ATP (P-L Biochemicals), 5.0 mM $MgCl_2$, 95 mM NaCl, 10 mM KCl, 1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–Tris, 0.36 mM NADH, 2 mM phosphoenolpyruvate, and 10 μL of dialyzed pyruvate kinase/lactate dehydrogenase (Sigma), pH 7.2. The K^+-dependent phosphatase activity [*p*-nitrophenylphosphatase (pNPPase)] was measured in a medium containing 30 mM histidine, 4 mM $MgCl_2$, 1 mM EGTA–Tris, 4 mM *p*-nitrophenyl phosphate, and 10 mM KCl (Lane et al., 1978), pH 7.4. The effect of antibody on these catalytic activities was recorded after prior incubation of 0.75–1.0 μg of (Na^+,K^+)-ATPase with varying amounts of protein (5–100 μg) at 37 °C for 20 min. The ATPase reaction was initiated by the addition of varying concentrations of ATP, Na^+, or K^+. The enzyme activity data were also corrected for endogenous or substrate-independent activity, which was less than 1%. The antibody M7-PB-E9 was purified by affinity chromatography in 0.14 M phosphate; therefore, extensive dialysis in either 25 mM Tris-HCl (pH 7.4) or 30 mM histidine was required to remove the phosphate. The effects of similarly dialyzed samples of antibody in the presence of additional sheep or rabbit IgG or ovalbumin were tested. The effects of sheep and/or rabbit IgG and ovalbumin samples and the dialysis buffer itself on enzyme activity and ouabain binding were also determined to be negligible before proceeding with each experiment.

Ouabain Binding. The effect of antibody on [^{3}H]ouabain binding was carried out at 37 °C essentially as described by Wallick & Schwartz (1974) using nine different binding conditions: (a) no ligands; (b) ATP; (c) Mg^{2+}; (d) MgATP; (e) NaMgATP; (f) NaMg; (g) NaKMgATP; (h) MgP_i; and (i) $NaMgP_i$. The ligand concentrations were 5 mM for the $MgCl_2$, Tris–ATP, and phosphate (P_i), 100 mM for NaCl, and 1 mM for KCl. The medium also contained 50 mM Tris-HCl, pH 7.4, and varying amounts of antibody or control immunoglobulin. The enzyme (5 μg) and antibody M7-PB-E9 or other immunoglobulins (sheep IgG, 25–100 μg) were preincubated for 30 min at 37 °C under the different conditions. Binding was initiated by adding [^{3}H]ouabain (1000 mCi/mmol) to obtain a final concentration of 1.0×10^{-7} M. The [^{3}H]ouabain binding was carried out for various times depending upon the ligands and the particular experiments. All values have been corrected for nonspecific binding as determined by the inclusion of 10^{-3} M unlabeled ouabain.

Enzyme Phosphorylation and Dephosphorylation. Steady-state phosphorylation of the (Na^+,K^+)-ATPase was done at 0 °C essentially as described by Post et al. (1969). The reaction mixture (0.2 mL) contained 5–10 μg of (Na^+,K^+)-ATPase and 30 mM histidine, 100 mM $NaCl_2$, 2 mM $MgCl_2$, and [γ-^{32}P]ATP [40 μM (1–2 Ci/mmol) or 1 μM (8 Ci/mmol)]. The phosphorylation was terminated after 10 s by adding 5 mL of cold 10% trichloroacetic acid (Cl_3CCOOH) 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 washing 3 times with 5 mL of the same solution. In order to compare relative rates of dephosphorylation, the enzyme was phosphorylated for 10 s as described above, and then EDTA (3.5 mM final concentration) alone and with Tris–ADP or KCl (5 and 0.10 mM, respectively) was added to the reaction mixture. The reaction was then terminated with Cl_3CCOOH after 5 s at 0 °C. To determine the effect of antibody on phosphorylation, 20–200 μg of antibody was incubated with enzyme for 45 min at 37 °C before the addition of [^{32}P]ATP.

Results

Antibody Binding Rates. Previous studies with three different purified monoclonal antibodies raised against the lamb kidney (Na^+,K^+)-ATPase indicated that all three were directed against the α subunit of the enzyme. Interestingly, titer curves showed that each of these three antibodies had a 2–3-fold higher titer value for the isolated, inactive α subunit than for the native enzyme against which it was raised. Two of the antibodies had little or no measurable effect on the (Na^+,K^+)-ATPase, but one of these antibodies (designated M7-PB-E9) was found to partially inhibit ATPase hydrolysis in a manner dependent upon the concentration of ATP in the assay medium (Ball et al., 1982). Because of its ability to inhibit enzyme activity, its interactions with (Na^+,K^+)-ATPase have been further characterized. Determination of the rate of antibody binding to plate-adsorbed protein shows that the initial rate of antibody binding to the isolated α subunit is about 10-fold faster (1.45 vs. 0.15 min^{-1}) than to the holoenzyme (Figure 1). The binding was done with antibody excess, and the rate plots show the binding to be multicomponent with an initial linear phase. To determine the rates of dissociation, the antibody was first bound to a limiting

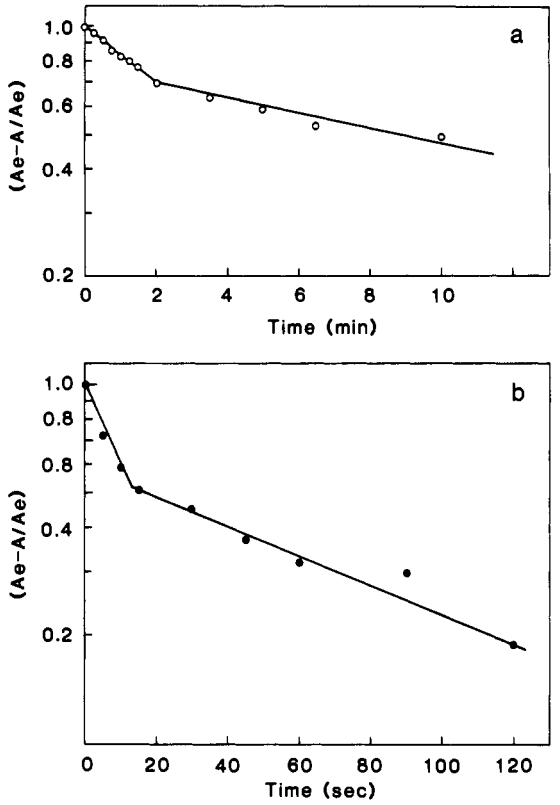

FIGURE 1: Rate of antibody M7-PB-E9 binding to (Na$^+$,K$^+$)-ATPase and isolated α subunit. (a) Open circles depict antibody binding to (Na$^+$,K$^+$)-ATPase. (b) Closed circles depict antibody binding to α subunit. Antibody binding was done at room temperature with excess antibody and with the antigen proteins adsorbed to plastic plates with 0.75 mg/mL solutions. The antibody was quantitated by using the ELISA assay with the anti-mouse antibody–β-galactosidase conjugate. Data are plotted as a pseudo-first-order rate according to the equation $\ln (A_e - A/A_e) = -(k_a a)t$, where A_e = antibody bound at 3 h (maximal binding), A = antibody bound at time t, and a is antibody concentration. $\ln (A_e - A/A_e)$ is plotted on a logarithmic scale vs. t. Each point was done in triplicate.

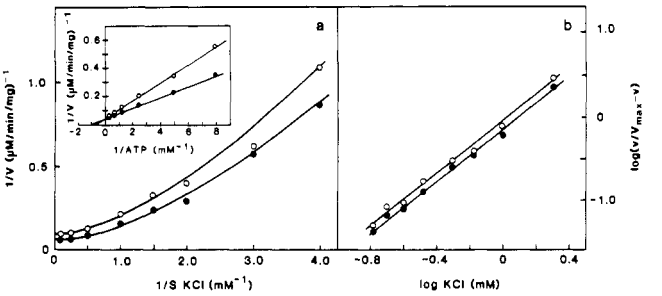

FIGURE 2: Effect of antibody M7-PB-E9 on the K$^+$ concentration dependence of (Na$^+$,K$^+$)-ATPase activity. (a) Closed circles represent the enzyme activity of untreated enzyme and open circles the activity in the presence of antibody. The enzyme was preincubated with antibody for 20 min in the reaction mixture as described under Materials and Methods. The reaction is initiated by adding KCl. The data are plotted as a Lineweaver–Burk plot. Each point is the average from two experiments done in duplicate. (b) Data from (a) are used to obtain Hill plots, with $n = 1.6$ for (●) enzyme only and (○) enzyme in the presence of antibody. The inset is the Lineweaver–Burk plot obtained for determination of the ATP concentration dependence of ATPase activity [see Ball et al. (1982)].

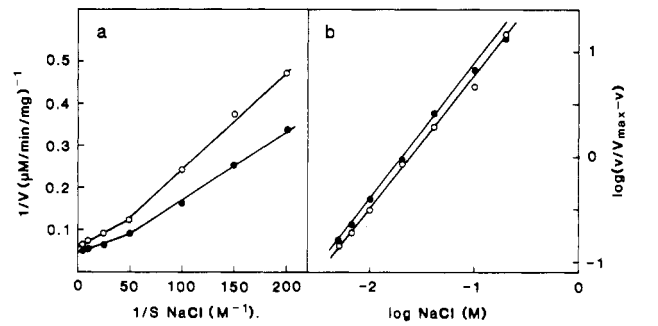

FIGURE 3: Effect of antibody M7-PB-E9 on the Na$^+$ concentration dependence of (Na$^+$,K$^+$)-ATPase activity. (a) Closed circles represent the enzyme activity of untreated enzyme and open circles the activity in the presence of antibody. The procedure is identical with that described previously, except that the assay was initiated with NaCl. (b) Data from (a) are used to obtain Hill plots, with $n = 1.2$ for untreated enzyme (●) and treated enzyme (○).

amount of titer plate adsorbed antigen [(Na$^+$,K$^+$)-ATPase or α], and then the unbound antibody was removed by washing the plate with buffer. The decrease in bound antibody upon exposure to antibody-free buffer was then determined. Under these conditions, over a 4-h time period there was little, if any, decrease in the amount of antibody bound to either protein. This slow rate of dissociation is that expected for high-affinity bivalent binding (Mason & Williams, 1980). Clearly, then, these association rate data are consistent with the previously observed titer value differences.

Effect of Antibody on ATP, K$^+$, and Na$^+$ Dependency of ATPase Activity. The antibody M7-PB-E9 inhibits up to 60% of the ATPase activity. A Lineweaver–Burk plot of enzyme activity as a function of varying ATP concentrations shows a linear relationship and suggests that the antibody acts as a partial competitive inhibitor with respect to ATP (see inset, Figure 2a). In contrast, a sigmoidal or nonlinear relationship with respect to K$^+$ concentration was obtained (Figure 2a). In addition, with an amount of antibody present such that an inhibition of about 30% occurred at 10 mM K$^+$, little change in the percentage of inhibition was observed at lower K$^+$ concentrations, and an essentially parallel curve of reduced activity was observed. Thus, the antibody appears to act as an uncompetitive or mixed inhibitor with respect to K$^+$. Hill plots of the K$^+$-dependent activity data (Figure 2b) give identical Hill coefficients of $n = 1.6$ in the presence or absence of antibody.

Nonlinear double-reciprocal kinetic plots were also obtained with respect to Na$^+$ concentration. Again with changing Na$^+$ concentrations, the extent of the antibody-caused inhibition did not vary significantly (Figure 3a), and the Hill coefficient of $n = 1.2$ was not affected by the antibody (Figure 3b). These results demonstrate a clear distinction between the manner in which the antibody affects the ATPase reaction with respect to its ATP dependence vs. Na$^+$ and K$^+$ dependence. The observed cooperativity of the K$^+$ and Na$^+$ cations on enzyme activity is consistent with the kinetic studies of others (Robinson & Flashner, 1979; Flashner & Robinson, 1979; Koepsell, 1978), and the values are nearly identical with the cooperativity values obtained by the recent direct ion binding studies of Matsui & Homareda (1982).

Antibody Effect on Partial Reactions of (Na$^+$,K$^+$)-ATPase Activity. The generally accepted reaction mechanism for the (Na$^+$,K$^+$)-ATPase reaction involves the ATP-, Na$^+$-, and Mg^{2+}-dependent formation of a so-called "E_1–P" conformation intermediate, its conversion to an "E_2–P" conformation with the release of Na$^+$, and then a K$^+$-dependent dephosphorylation of E_2–P to enzyme + P_i. Several steps in this scheme can be studied separately. The steady-state level of formation of the phosphoenzyme intermediate was measured after 10 s of [^{32}P]ATP phosphorylation in the presence of Na$^+$ and Mg^{2+} at 0 °C. This phosphorylated enzyme was then dephosphorylated for 5 s following the addition of ethylenediaminetetraacetic acid (EDTA) (3.5 mM to give low Mg^{2+}) and 5

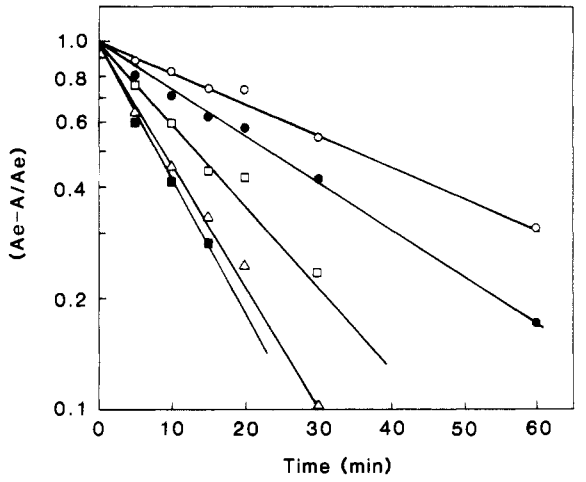

FIGURE 4: Effect of antibody on binding of [^{3}H]ouabain to (Na^+,K^+)-ATPase. The values given are for Mg^{2+} (5.0 mM) binding conditions: (○) enzyme plus sheep IgG; (●) enzyme only; (□) enzyme plus antibody. For Mg^{2+}ATP conditions (5.0 mm): (△) enzyme only; (■) enzyme plus antibody M7-PB-E9. These data are plotted as pseudo-first-order plots according to the equation $\ln (A_e - A/A_e) = -(kI + k')t$, as described by Wallick et al. (1977), where A and A_e represent the amount of [^{3}H]ouabain bound to the enzyme at time t and at equilibrium, respectively, k and k' are the apparent forward and dissociation rate constants, respectively, and I is ouabain concentration. Data are plotted as $\ln (A_e - A/A_e)$ on a logarithmic scale vs. t.

mM ADP (for ADP ⇌ ATP exchange) or EDTA and 0.1 mM KCl. The antibody decreases the level of phosphoenzyme formed, and the extent of the inhibition appears to be somewhat dependent upon ATP concentration (Table I). In contrast, the antibody had no effect on the K^+-dependent dephosphorylation step or on the ADP-sensitive dephosphorylation. In addition, the enzyme activity with the artificial substrate *p*-nitrophenyl phosphate (pNPP), which is considered to be a measure of the K^+-dependent dephosphorylation step of the overall catalysis process, was not affected by the antibody (data not shown). Clearly, the antibody affects the phosphorylation step only.

Antibody Effects on Ouabain Binding. It is well-known that the affinity of (Na^+,K^+)-ATPase for ouabain is greatly influenced by the ligands present in the reaction medium, and little binding occurs in their absence (Wallick et al., 1977). A relatively slow rate of ouabain binding occurs when only Mg^{2+} is present, and the fastest rate is in the presence of Mg^{2+} and P_i. The presence of antibody had no significant effect on the equilibrium binding of ouabain with several different binding conditions used, and it did not alter the rates of binding except in the presence of Mg^{2+} alone (Table II). When Mg^{2+} only was present, the antibody caused a substantial stimulation of ouabain binding. The $t_{1/2}$ values for [^{3}H]ouabain binding in the presence of Mg^{2+}, Mg^{2+} plus sheep IgG, and Mg^{2+} plus antibody M7-PB-E9 were 23, 35, and 13 min, respectively (Figure 4). The $t_{1/2}$ for Mg^{2+}ATP conditions was 9 min with little additional effect with added antibody (M7-PB-E9). This antibody-caused stimulation of ouabain binding requires Mg^{2+}. The binding of antibody alone to the enzme did not stimulate ouabain binding. In these respects, the antibody M7-PB-E9 affects ouabain binding to (Na^+,K^+)-ATPase in a manner similar to that of ATP. This stimulation also appears to be specific for the antibody M7-PB-E9 since the α subunit directed monoclonal antibodies M8-P1-A3 and M10-P6-B7 had no effect on the ouabain binding rates and nonspecific sheep IgG fractions somewhat reduced the binding rate in the presence of Mg^{2+} alone.

Table I: Effect of Antibody M7-PB-E9 on Steady-State Phosphoenzyme Levels[a]

[ATP] (μM)	steady-state phosphorylation levels (%)	
	control	plus antibody
40	100 (1172 pmol/mg)	84 ± 8
1	100 (411 pmol/mg)	71 ± 5

conditions	phosphoenzyme sensitivity to dephosphorylation (%)	
	control	plus antibody
phosphorylation (10 s) additions (5 s)	100	100
EDTA	76.5	71.8
EDTA + 5 mM ADP	19.9	18.2
EDTA + 0.1 mM KCl	35.1	34.5

[a] Enzyme fractions (5–10 μg) were phosphorylated with 40 or 1 μM [γ-^{32}P]ATP for 10 s at 0 °C after a 45-min preincubation of enzyme with antibody M7-PB-E9 or sheep IgG at 37 °C as described under Materials and Methods. The dephosphorylation reactions were terminated after 5 s at 0 °C following a 10-s phosphorylation. Values are given as percentages relative to the initial phosphorylation levels for the controls and experimental samples. All values given are results from two to six experiments each done in triplicate. Except where indicated, the standard deviation for results was <5%.

Table II: Effect of Monoclonal Antibody on Ouabain Binding

ligands present	equilibrium binding[a] (relative % bound)	
	control	plus antibody
Mg^{2+}	100	127
Mg^{2+}ATP	100	104
$Na^+K^+Mg^{2+}$ATP	100	101
$Mg^{2+}P_i$	100	105

ligands present	relative rates of binding[b] (%)	
	control	plus antibody
(a) none	2.0	4.9
(b) ATP	2.4	
(c) Mg^{2+}	100	243
(d) Mg^{2+}ATP	264	316
(e) $NaMg^{2+}$	379 (plus ATP)	0.6 (no ATP)
(f) $Na^+K^+Mg^{2+}$ATP	50	47.5
(g) $Mg^{2+}P_i$	100	102

[a] For determination of equilibrium binding, the (Na^+,K^+)-ATPase was preincubated with antibody M7-PB-E9 (100 μg) or nonspecific sheep IgG (as control) for 30 min at 37 °C under the specific ligand conditions, and then [^{3}H]ouabain was added to 1×10^{-7} M as described under Materials and Methods. Equilibrium binding times were 4 h for Mg^{2+} and Mg^{2+}ATP conditions and 2 h for $Na^+K^+Mg^{2+}$ATP and $Mg^{2+}P_i$ conditions, with approximately 1969 pmol of [^{3}H]ouabain bound/mg of protein for controls except under Mg^{2+} conditions where 1028 pmol/mg of protein was obtained for 4–5-h binding time. Values are given as the percent relative to the control for each binding condition.
[b] The relative rates of binding were compared by determining [^{3}H]ouabain binding to the enzyme at a single time point, which varied depending upon ligand conditions: (a) none and (b) ATP alone had binding times of 30 min, and values are given relative to the amount of [^{3}H]ouabain bound in the presence of Mg^{2+} alone; (c) Mg^{2+}, (d) Mg^{2+}ATP, and (e) $NaMg^{2+}$ plus ATP or plus antibody all had 15-min binding times, and rates are compared to 15-min binding with Mg^{2+} only conditions; and (f) $Na^+K^+Mg^{2+}$ATP and (g) $Mg^{2+}P_i$ had 5-min binding times and are compared to the amount bound with $Mg^{2+}P_i$ present. Controls all had nonspecific sheep IgG present (100 μg), and determinations are the average of two to three experiments each done in triplicate.

However, antibody binding did not fully mimic ATP binding, since Na^+ stimulates the rate of ouabain binding in the presence of Mg^{2+}ATP, and in the presence of Mg^{2+} and an-

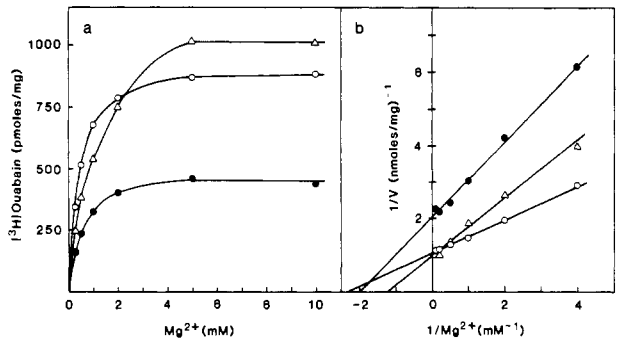

FIGURE 5: Effect of antibody and ATP on the initial rate of [^{3}H]-ouabain binding to (Na^+,K^+)-ATPase in the presence of varying Mg^{2+} concentrations. The initial rates of ouabain binding are expressed as picomoles bound per milligram of protein per 30 min and plotted as a Lineweaver–Burk plot. The closed circles represent binding in the presence of Mg^{2+}, the open circles Mg^{2+} plus antibody, and the open triangles Mg^{2+} plus 5 mM Tris–ATP.

tibody, Na^+ virtually abolishes ouabain binding (Table II). This may be analogous to the Na^+-caused decreases in ouabain binding under Na^+Mg^{2+} or $Na^+Mg^{2+}P_i$ conditions (binding 1% and 13% of controls, respectively). In addition, the initial rate of ouabain binding to the enzyme as a function of the Mg^{2+} concentration was not the same in the presence of ATP as with antibody present. Double-reciprocal plots of the rate data indicate that the apparent affinity or $K_{0.5}$ for the Mg^{2+} stimulation of ouabain binding was decreased from 0.40 to 0.90 mM Mg^{2+} by 5 mM ATP, while the V_{max} was increased (Figure 5). In the presence of antibody, the apparent affinity for Mg^{2+} was unaltered, or perhaps slightly increased. Therefore, at low Mg^{2+} concentrations, the antibody stimulated ouabain binding more effectively than did ATP. Presumably, free Mg^{2+} can bind to the enzyme when the antibody is present, while with an excess of ATP, Mg^{2+} binds as a Mg^{2+}–ATP complex.

Ligand Effects on Antibody Binding to Holoenzyme. Antibody binding rate studies were also done by using a relatively low (10 nM) concentration of antibody (titer value = 8 nM) in order to determine possible sensitivities of antibody binding to ligand-induced conformational changes in the holoenzyme. The data revealed no differences in the rate of antibody binding to the plate-absorbed enzyme in the presence or absence of 5 mM Mg^{2+}ATP in Tris–saline binding buffer (data not shown). In addition, there was no difference in the rate of antibody binding to holoenzyme in the absence of ligands, or with $MgCl_2$ or only NaCl present. Dissociation experiments were also done in the presence and absence of 5 mM MgATP, and no difference in the rate of antibody dissociation from the enzyme was observed (data not shown). These results suggest that with the plate-bound enzyme, the Mg–ATP complex does not block antibody binding or stimulate antibody dissociation. Indeed, kinetic studies showed that under standard ATPase activity conditions and antibody excess, preincubation of the antibody with the enzyme before the addition of ATP only increased the extent of inhibition of ATPase activity by about 15–20%. These binding studies did not demonstrate any ligand-induced effects on antibody binding.

Discussion

We have recently obtained and partially characterized several monoclonal antibodies which are directed against the α subunit of the lamb (Na^+,K^+)-ATPase (Ball et al., 1982). In the present experiments, the interactions of one of these antibodies (M7-PB-E9) with (Na^+,K^+)-ATPase have been studied in some detail.

ELISA antibody binding studies have shown that this antibody has a faster rate of binding to the isolated α subunit than to the native enzyme and that antibody binding to the plate-adsorbed enzyme is not affected by different ligand conditions which are believed to induce conformational changes in the enzyme. This antibody also acts like a partial competitive inhibitor with respect to ATP and inhibits enzyme phosphorylation but not its dephosphorylation. It does not appear to affect Na^+ and K^+ binding to the enzyme or their regulation of enzyme activity, and it does not perturb the positive interactions between the nonequivalent Na^+ or K^+ binding sites. In the presence of Mg^{2+}, the antibody M7-PB-E9 can stimulate ouabain binding to the enzyme almost as well as ATP, and like ATP, it has no effect on ouabain binding in the absence of Mg^{2+}.

The higher titer value of this antibody toward the inactive α subunit rather than for the active enzyme appears to result from differences in the rate of binding, not in the rate of dissociation. However, the two binding rate curves show multiphasic curves, and this may be due to the saturation of binding sites with the bivalent antibody or to the existence of different forms of the antigens in each fraction. The lower "apparent" affinity of the antibody for the holoenzyme may be caused by conformational differences or steric constraints imposed upon the epitope due to the enzyme complex and its lipids which may not exist for the isolated α subunit. Alternatively, it may be due to anomalies caused by binding of the proteins to the solid phase. However, our rabbit holoenzyme-directed antibodies were also found to have about a 2-fold higher affinity for the α subunit vs. holoenzyme when competition binding studies were done in solution (Ball & Schwartz, 1982).

The present study does show that a single antibody can affect specific functional parameters of the enzyme. These results cannot be due to the cumulative effects of many different antibody bindings at many different antigenic sites. The ATP and *p*-nitrophenyl phosphate (pNPP) hydrolysis studies and the phosphorylation studies suggest that antibody M7-PB-E9 alters ATP binding, or enzyme phosphorylation without altering the dephosphorylation steps. Askari (1974) and others (Ball & Schwartz, 1982; Glynn et al., 1974) have previously observed with rabbit polyclonal antibodies an apparent decreased sensitivity of the pNPPase activity to antibody inhibition relative to the ATPase activity. Askari (1974) has interpreted these results to suggest the existence of antigenically distinct portions of the catalytic site or perhaps a distinct separate site for the pNPPase activity. The antibody M7-PB-E9 has unambiguously demonstrated an immunochemical distinction between these two activities. This suggests the existence of a single antigenic site to which the antibody binds and then alters ATP binding. These studies are also consistent with the existence of a single active site since both the K^+-dependent dephosphorylation step and the pNPPase activity are not inhibited. It is interesting that an analogous inhibition of the ATPase activity but not the pNPPase activity has also been observed when the enzyme is subjected to chemical modification by the sulfhydryl group directed thimerosol [sodium (ethylmercuri)thiosalicylate] (Henderson & Askari, 1976, 1978; Hansen et al., 1979).

The ouabain binding studies further suggest that the antibody is binding at or near the ATP site. In the presence of Mg^{2+}, the antibody stimulates ouabain binding almost as well as ATP itself, but far less than P_i. Like ATP, the antibody binds to the enzyme but has no effect on ouabain binding in the absence of Mg^{2+}. However, the antibody also acts

somewhat like P_i in that Na^+ strongly inhibits ouabain binding in the presence of Mg^{2+} plus antibody while Na^+ stimulates ouabain binding in the presence of Mg^{2+} and ATP.

Clearly, though, these studies cannot determine if the antibody is binding directly to the ATP binding site or if the effects of the antibody are due to a steric effect by binding in the proximity of the ATP site or to antibody-induced conformational changes transmitted to distant sites. Since the antibody only partially inhibits the ATPase activity, and since both the antibody-caused inhibition of the ATPase activity and the antibody binding to the plate-adsorbed enzyme are largely unaffected by the prior presence of Mg^{2+}ATP, it would appear that the antibody alters the enzyme's affinity for ATP without blocking the ATP site. Alternatively, since the enzyme is purified in a particulate, vesicular form with the enzyme presumably randomly oriented to both sides of the membrane, the partial inhibition may result from the inaccessibility of some of the antigenic sites to the antibody.

It is interesting that these antibody effects result from its binding to what is presumed to be a single site on the enzyme. There is some controversy concerning the possible existence of two ATP binding sites, an ATP phosphorylation site with a K_m of 0.3–3 μM and another ATP regulatory site with a K_m of 0.2–2 mM (Robinson & Flashner, 1979; Cantley et al., 1978a). Ouabain binding (Dr. E. T. Wallick, unpublished results) is then also regulated by high-affinity ATP binding. More recently, Moczydlowski & Fortes (1981a,b), using a fluorescent ATP derivative probe, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphoshate, have results that suggest the existence of a single ATP site which exhibits a high or low affinity for ATP depending on the ligands present. Studies by Cantley et al. (1978b) with the inhibitor vanadate also support this model.

However, Askari & Huang (1982) have recently reported evidence for the existence of a second, or low-affinity, noncatalytic site for ATP which affects enzyme dephosphorylation. In addition, Gupte & Lane (1983), using the fluorescent probes *N*-(1-pyrenyl)maleimide (Malpyrene) and 6-(4-maleimidoanilino)naphthalene-2-sulfonic acid (MalANS) with the lamb kidney enzyme, have data consistent with the concept of two distinct high-affinity ATP sites (in the presence of Mg^{2+} and Na^+), one which appears to be involved in enzyme phosphorylation and another which regulates ouabain binding, but they do also offer alternative explanations for their data.

The immunological studies clearly demonstrate for the first time the possible specificity of antibody effects on (Na^+,K^+)-ATPase function. This antibody appears to bind to a region of the enzyme such that it alters ATP binding to the enzyme and partially substitutes for ATP in affecting a regulatory conformational change in the enzyme that stimulates ouabain binding. These studies also demonstrate that Mg^{2+} is required for this effect and, as previously suggested by Anner et al. (1976), that a conformational change, but not necessarily phosphorylation of the enzyme, is required for the ATP stimulatory effect on ouabain binding.

Added in Proof

After this paper was submitted, Schenk & Leffert (1983) reported monoclonal antibodies that inhibit the rat kidney (Na^+,K^+)-ATPase.

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Registry No. ATPase, 9000-83-3; ouabain, 630-60-4.

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Two Populations of Phospholipids Exist in Sarcoplasmic Reticulum and in Recombined Membranes Containing Ca-ATPase†

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ABSTRACT: Phosphorus nuclear magnetic resonance spectra of sarcoplasmic reticulum membranes from rabbit muscle and of recombined membranes containing the calcium-dependent adenosinetriphosphatase (Ca-ATPase) of sarcoplasmic reticulum reveal two distinguishable, overlapping resonances. One resonance resembles a normal phospholipid bilayer resonance, and the other is much broader. The broader component is not seen in protein-free phospholipid vesicles. In recombined membranes of the Ca-ATPase, the intensity found in the broad component was proportional to the concentration of protein in the vesicles. The two-component spectra are interpreted to arise from at least two different domains of phospholipids, one of which is motionally restricted by the Ca-ATPase. Phospholipids exchange between these two domains at a rate less than 10^3 s^{-1}. A model for protein–lipid interactions in membranes containing the Ca-ATPase is proposed in which some of the phospholipid head groups of the membrane interact directly with the protein.

Since the proposal of the fluid-mosaic model for membrane structure (Singer, 1974), much effort has been expended to determine how membrane lipids interact with proteins embedded in the lipid bilayer. Previous studies measured the effect of membrane proteins on spin-labeled or deuterated lipid probes located in the hydrophobic interior of the phospholipid bilayer. The results obtained were dependent upon the exact location and composition of the probe, and the time scale of the measurement. Little information is available on the behavior of the membrane surface in the presence of membrane proteins.

Sarcoplasmic reticulum isolated from rabbit skeletal muscle is an ideal system in which to study protein–lipid interactions. Over 90% of the protein of the light fraction of sarcoplasmic reticulum is the calcium-dependent adenosinetriphosphatase (Ca-ATPase)[1] (Meissner, 1975). The structure and function of this transmembrane pump have been extensively studied (Tada et al., 1978; Ikemoto, 1982). Furthermore, the Ca-ATPase can be easily solubilized in detergents and examined free of lipids (Dean & Tanford, 1978) or reconstituted into a defined lipid environment (Bennett et al., 1978a).

In a previous study (Albert et al., 1981), we measured interactions between phospholipids and the Ca-ATPase protein of sarcoplasmic reticulum by using phosphorus NMR. Phosphorus NMR has several advantages over deuterium NMR and electron spin resonance (ESR) in the study of protein–lipid interactions. First, phosphorus-31 is 100% naturally abundant, and a foreign probe does not have to be introduced. Second, phosphorus NMR measures the properties of the phospholipid head groups which likely are important to biological membrane function (Yeagle, 1978; Seelig, 1978). In our earlier study, using an indirect method (Albert et al., 1981), some of the phospholipid head groups in a crude

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; Ca-ATPase, calcium-dependent adenosinetriphosphatase; FID, free induction decay of the ^{31}P NMR signal; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CSA, chemical shift anisotropy.

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