Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 246-253

Immunochemical Evidence that the FITC-labeling Site on Na^+,K^+-ATPase is Not the ATP Binding Site

W.J. Ball, Jr. and M.L. Friedman

Department of Pharmacology and Cell Biophysics,
University of Cincinnati College of Medicine,
Cincinnati, Ohio 45267-0575

Received August 25, 1987

The covalent labeling of the α subunit of lamb kidney Na^+,K^+-ATPase by fluorescein 5'-isothiocyanate at Lys-501 has generally been assumed to occur at the ATP binding site. We have found that the peptide sequence ^{496}HLLVMKGAPER^{506} serves as the antigenic determinant for monoclonal antibody M8-P1-A3. This antibody binds to both native and FITC-labeled enzyme and while this epitope undergoes ligand-induced changes these changes are not involved in either enzyme function or the $E_1 \rightleftharpoons E_2$ conformational changes monitored by FITC-fluorescence intensity.

Na^+,K^+-ATPase is a membrane enzyme, composed of two subunits, the α subunit (~100,000 M_r) and the β subunit (~50,000 M_r), whose primary function is the maintenance of K^+ and Na^+ levels in the cell. This enzyme belongs to a class of ATP-driven ion transporters, designated by Racker (1) as E_1E_2 pumps, whose functioning involves the cycling of the enzymes between a series of different conformations. The fluorescent probe fluorescein 5'-isothiocyanate (FITC) has been used extensively to study these ligand-induced changes. FITC has been found to inactivate the enzyme's ATPase activity by labeling a specific lysine residue in a region of the 100 K_D protein which is highly conserved among the E_1E_2-ATPase pumps. Because FITC reacts with a 1:1 stoichiometry to ATP binding and ATP blocks FITC labeling, the site of labeling is generally thought to be a portion of the ATP binding site (2). We have found that monoclonal antibody M8-P1-A3 binds to this region of α. The effect of bound antibody on enzyme function and ligand-induced conformational changes has

0006-291X/87 $1.50

been investigated. The effects of these ligands on antibody binding to Na^+,K^+-ATPase have also been determined.

EXPERIMENTAL PROCEDURES

Enzyme preparation and assays. The Na^+,K^+-ATPase was prepared from frozen lamb kidneys as previously described by Lane et al (3). The Na^+,K^+-ATPase activity at 37°C was measured at 340 nm using the spectrophotometric, linked enzyme assay system of Schwartz et al (4) while the K^+-dependent p-nitrophenylphosphatase activity was determined by continuous measurement at 410 nm (5). [^{3}H]-ouabain binding to the enzyme was determined at 37°C essentially as described by Wallick and Schwartz (6). The labeling of Na^+,K^+-ATPase with FITC (Molecular Probes, Inc.) has been described in our previous work (5), while the fluorescence intensity measurements were measured at 520 nm as described by Hegyvary and Jorgensen (7).

Antibody M8-P1-A3 preparation and binding determinations. This antibody was raised against the lamb kidney Na^+,K^+-ATPase and its isolation, purification and initial characterization was reported previously by Ball et al. (8). The indirect solid-surface adsorption (ELISA) assay used to quantitate antibody binding has been described in detail (8) but we have modified the procedure somewhat. In this procedure antibody and enzyme were combined for 1-2 h and then the enzyme and any antibody-enzyme complex formed were removed from solution by centrifugation at 150,000 x g for 1 h. The unbound M8-P1-A3 (in the supernatant) was then exposed to either untreated microtiter plates (Cooke flexible plates), plates previously coated with enzyme (± 0.1% SDS), or synthetic peptide-BSA conjugates. M8-P1-A3 binding to these plates was then quantitated using a β-galactosidase sheep anti-mouse IgG $F(ab')_2$ conjugate as second antibody. Solution phase binding of M8-P1-A3 to the enzyme under various ligand conditions (Na^+; K^+; Mg^{2+}; $Mg^{2+}P_i$ and Mg^{2+}ATP, all at 5 mM) was also examined.

RESULTS

A collection of five monoclonal antibodies raised to the lamb kidney Na^+,K^+-ATPase (9) were tested for their ability to recognize a series of synthetic peptides (11-15 amino acid residues in length) representing various portions of α (10). One antibody, M8-P1-A3, was found to bind to the sequence ^{496}HLLVMKGAPER^{506} conjugated to bovine serum albumin (BSA) (Fig. 1, peptide 6). This sequence represents a soluble tryptic digestion fragment of α that has been isolated, sequenced and shown to be the covalent labeling site for FITC (2,11). Because this region may comprise a portion of the ATP binding site of Na^+,K^+-ATPase, the binding of M8-P1-A3 to this region was further characterized. Figure 2 shows that half-maximal binding of M8-P1-A3 to holoenzyme and peptide 6-BSA conjugate was achieved at approximately 7 and 85 nM antibody, respectively. While the affinity of the antibody for the peptide was about 12-fold lower than for enzyme, the maximum levels of bound antibody

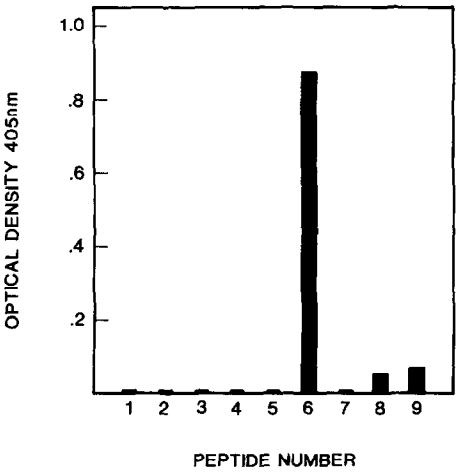

Fig. 1. Determination of Monoclonal Antibody M8-P1-A3 Binding to Synthetic Peptides.
Antibody binding to peptide-BSA conjugates was determined using the solid-surface adsorption assay and a β-galactosidase sheep anti-mouse IgG $F(ab')_2$ conjugate as second antibody. Monoclonal antibody concentration was 1 μM. The sequence regions for each of the α peptides were: (1) 1-12; (2) 16-30; (3) 111-122; (4) 303-314; (5) 366-377; (6) 496-506; (7) 823-833; (8) 932-943; and (9) 1,003-1,013.

achieved were similar. Competition binding studies (Fig. 3) then demonstrated that antibody binding to holoenzyme "in solution" effectively prevented antibody binding to both plate-adsorbed enzyme and peptide 6-BSA conjugate. These

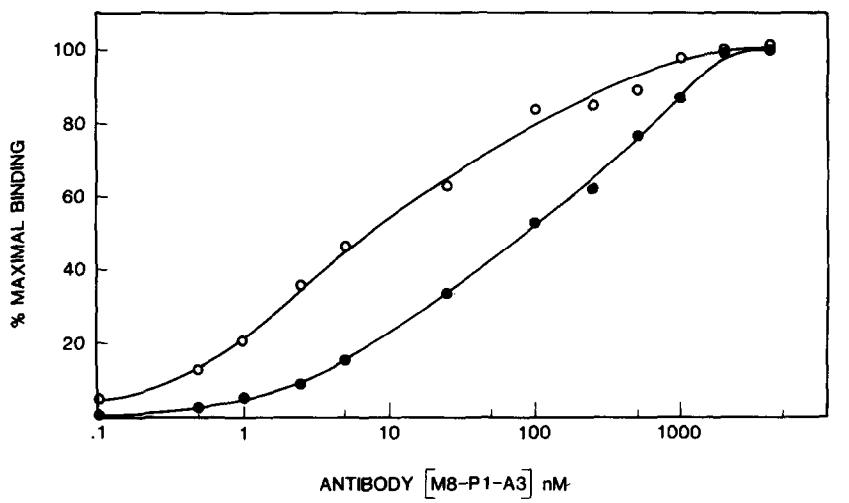

Fig. 2. Determination of M8-P1-A3 Binding to Antigen as a Function of Antibody Concentration.
Open circles (o) and closed circles (•) represent antibody binding to Na^+,K^+-ATPase and peptide 6-BSA conjugate, respectively. All values are given as the percentage of antibody binding relative to the maximum amount bound to each particular antigen. Antibody binding to antigen was detected using a solid-surface adsorption assay. Antigen was adsorbed to the plates at 50 μg/well (Na^+,K^+-ATPase) and 5 μg/well (peptide 6-BSA conjugate) and bound antibody quantitated using β-galactosidase sheep anti-mouse IgG $F(ab')_2$ conjugate.

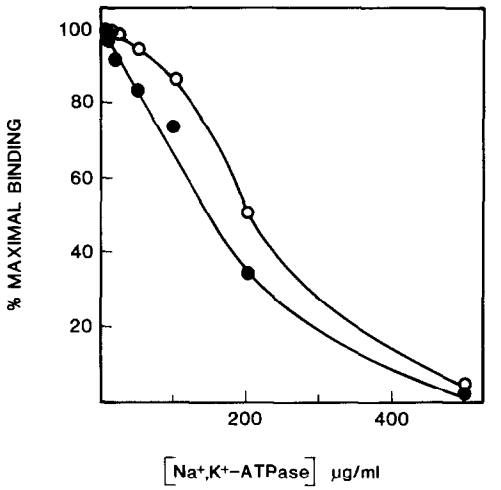

Fig. 3. Competition Binding Study.
Open circles (o) and closed circles (•) show decrease in M8-P1-A3 binding to plate adsorbed Na^+,K^+-ATPase and peptide 6-BSA conjugate, respectively, after exposure to varying concentrations of Na^+,K^+-ATPase. Antibody concentration was 10 nM. Plate adsorbed antigen concentrations were 50 µg/well, Na^+,K^+-ATPase and 5 µg/well, peptide 6-BSA conjugate. Binding of antibody to Na^+,K^+-ATPase in solution phase occurred before exposing antibody to either solid-surface bound peptide 6-BSA conjugate or Na^+,K^+-ATPase.

results showed that the antibody recognized "native" Na^+,K^+-ATPase and that this binding corresponded to antibody recognition of the plate-adsorbed antigens. Having established antibody binding to both native enzyme and peptide conjugate, antibody effects on enzyme function were determined. In these studies a high ratio of antibody to enzyme (>50:1) was used because our "affinity" or binding titer data (9) and competition binding curves showed that the relative order of antibody affinity was; plate-adsorbed isolated α > plate-adsorbed holoenzyme > native holoenzyme. Excess antibody was found to have no effect on Na^+,K^+-ATPase or K^+-dependent p-nitrophenylphosphatase (pNPPase) activity. M8-P1-A3 also had no effect on the rate of ouabain binding under various ligand conditions (Mg^{2+}; Mg^{2+}ATP; $K^+Na^+Mg^{2+}$ATP; and $Mg^{2+}P_i$, data not shown).

In addition, since the antibody was binding to the same region of α as FITC, its effects on FITC fluorescence were investigated. First, competition binding studies were done, as before, which showed that M8-P1-A3 bound to the FITC-modified enzyme exactly as it did to unlabeled enzyme. Next,

fluorescence spectroscopy measurements showed that antibody binding to the enzyme (in Tris-HCl buffer) had no effect on the fluorescence intensity of the bound probe. Further, the K^+, $Mg^{2+}P_i$ and Mg^{2+}·ouabain dependent decreases in FITC fluorescence intensity and their reversal by Na^+ were not effected by bound antibody. These results made it clear that M8-P1-A3 was neither altering enzyme function, nor perturbing the environment of the fluorescent moiety of FITC and its responses to the addition of regulatory ligands (data not shown).

The epitope for M8-P1-A3, therefore, appeared to be an exposed region (since it is antibody accessible) that is not involved in the enzyme's conformational changes. This concept was tested by a series of experiments in which antibody and enzyme were combined for 1 h either before or after the addition of ligands and then the level of unbound antibody was determined. Using conditions where approximately 70-75% of M8-P1-A3 was enzyme bound, we found that the addition of Na^+, K^+, Mg^{2+}, $Mg^{2+}P_i$ or Mg^{2+}ATP to the binding solution had essentially no effect on the level of bound antibody. In contrast, if the enzyme and ligands were combined prior to antibody addition, we found that Na^+ and K^+ had little effect but Mg^{2+}, $Mg^{2+}P_i$ and Mg^{2+}ATP caused a reduction in binding from 75% to 35, 18 and 20%, respectively. These data demonstrated that M8-P1-A3 binding to the enzyme was sensitive to the enzyme's conformational state but that bound M8-P1-A3 was not displaced from the enzyme by these conformational changes. Figure 4 clearly illustrates this binding sensitivity. It shows the decrease in free antibody as the concentration of enzyme, under different ligand conditions, is increased. To achieve 50% antibody binding in the presence of $Mg^{2+}P_i$ required a four-fold higher concentration of enzyme than was required in the absence of added ligands.

DISCUSSION

These data demonstrate that the sequence region 496-506 of α, which represents the epitope for monoclonal antibody M8-P1-A3, undergoes some ligand-induced conformational or tertiary structural rearrangements which

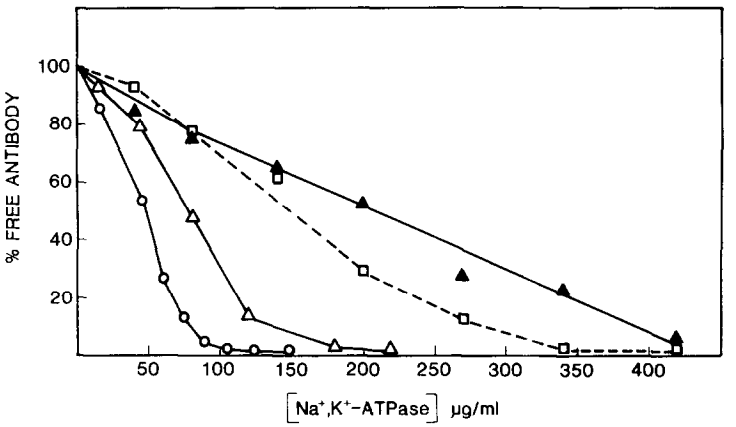

Fig. 4. Effect of ligands on antibody binding to Na^+,K^+-ATPase. The decreasing curves show the ability of Na^+,K^+-ATPase under various ligand conditions to compete for M8-P1-A3 binding to plate adsorbed (SDS-treated) enzyme. Open circles (o) show Na^+,K^+-ATPase concentrations in Tris-HCl buffer only; Open triangles (Δ) buffer plus Mg^{2+}; Open squares (□) buffer plus Mg^{2+}ATP; and Closed triangles (▲) buffer plus $Mg^{2+}P_i$. Antibody (1.5 µg/ml) and enzyme binding was done for 1 hr at 4°C before the centrifugation step was done.

alter M8-P1-A3 binding. These changes are not sufficient, however, to displace bound antibody. In addition, we find that bound antibody has no effect on either enzyme function, or the ligand-induced changes which alter the fluorescence intensity of enzyme-linked FITC. Further, we find it of interest that M8-P1-A3 cross-reacts with denatured rat kidney Na^+,K^+-ATPase but not with the native rat enzyme (9). The amino acid sequences of these enzymes are identical in this region, while their apparent tertiary configurations are not. Nevertheless, these two enzymes have similar affinities for ATP (12).

We therefore conclude that this region is not involved in either ATP binding or hydrolysis. However, given the interactions between ATP and FITC, the Lys-501 residue must reside in the proximity of the adenine-binding site (and fluorescein site) in order for it to serve as the reactive site for the thiocyanate group of FITC. The inactivation of the ATPase activity then appears to result from irreversibly fixing FITC in a position to sterically block the ATP-binding region rather than from the modification of the lysine group. This hypothesis also is consistent with the fact that eosin, a fluorescein derivative, is a competitive inhibitor with respect to ATP, and it

inhibits ATPase activity without chemically modifying the enzyme (13). It seems likely that these two probes bind at the same region of α, yet eosin's emission spectra undergoes a red shift upon binding, while FITC does not. This suggests that upon labeling the Lys residue FITC is repositioned and the differences in the two probes' functional effects results from this difference in their specific locations. This is in contrast to the 5'iodoacetamido-fluorescein (5'-IAF) derivative which requires at least a 20-fold higher concentration to label Na^+,K^+-ATPase and clearly reacts with several different -SH groups (14). Though it shows fluorescence intensity changes analogous to those of FITC, its labeling is not blocked by ATP and enzyme activity is not effected by the covalent labeling (15,16). These results emphasize the difficulties in using chemical modification techniques to identify functionally important regions of proteins.

Currently the sequence regions involved in ATP binding have not been established but two ATP analogues have been shown to react at amino acid residues considerably distant from the Lys-501. The alkylating ATP analogue γ[4-(N-2-chlorethyl-N-methylamino)] benzylamide-ATP (ClRATP) reacts at Asp-710 (17) and 5'-(p-fluorosulfonyl)benzoyl adenosine (FSBA) at the Cys-656 and Lys-719 of α (18). These regions may not be any more likely to represent actual portions of the ATP binding site but they are additional regions that require investigation.

ACKNOWLEDGEMENTS

We thank Purabi Dey and Chuck Loftice for their excellent technical assistance. This work was supported by NIH grants T32-HL-07382 (MLF) and RO1-HL-32214 (WJB). WJB is an Established Investigator of the American Heart Association.

REFERENCES

1. Racker, E. (1985) Reconstitutions of Transporters and Receptors, and Pathological States, Academic Press, New York, pp. 87-103.

2. Farley, R.A., Tran, C.M., Carilli, C.T., Hawke, D, and Shively, J.E. (1984) J. Biol. Chem. 259, 9532-9535.
3. Lane, L.K., Potter, J.D, and Collins, J.H. (1979) Prep. Biochem. 9, 157-170.
4. Schwartz, A., Allen, J.C. and Harigaya, S. (1969) J. Pharmacol. Exp. Ther. 168, 31-41.
5. Ball, W.J. (1986) Biochemistry 25, 7155-7162.
6. Wallick, E.T. and Schwartz, A. (1974) J. Biol. Chem. 249, 5141-5147.
7. Hegyvary, C. and Jorgensen, P.L. (1981) J. Biol. Chem. 256, 6296-6303.
8. Ball, W.J., Schwartz, A. and Lessard, J.L. (1982) Biochim. Biophys. Acta 719, 413-423.
9. Ball,W.J. and Lane, L.K. (1986) Biochim. Biophys. Acta 873, 79-87.
10. Ball, W.J. and Loftice, C.D. (1987) In press.
11. Kirley, T.L., Wang, T., Wallick, E.T. and Lane, L.K. (1985) Biochem. Biophys. Res. Commun. 130, 732-738.
12. Periyasamy, S.M., Huang, W.H. and Askari, A. (1983) Comp. Biochem. Physiol. 76B(3), 449-454.
13. Skou, J.C. and Esmann, M. (1981) Biochim. Biophys. Acta 647, 232-240.
14. Steinberg, M. and Kirley, T. Unpublished results.
15. Kapakos, J.G. and Steinberg, M. (1986) J. Biol. Chem. 261, 2084-2089.
16. Kapakos, J.G. and Steinberg, M. (1986) J. Biol. Chem. 261, 2090-2095.
17. Ovchinnikov, Yu.A., Dzhandzugazyan, K.N., Lutsenko, S.V., Mustayev, A.A. and Modyanov, N.N. (1987) FEBS Letters 217, 111-116.
18. Ohta, T., Nagano, K. and Yoshida, M. (1986) Proc. Nat'l Acad. Sci. USA 83, 2071-2075.