The Epitope for the Inhibitory Antibody M7-PB-E9 Contains Ser-646 and Asp-652 of the Sheep Na^+,K^+-ATPase α-Subunit†

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ABSTRACT: The binding of monoclonal antibody M7-PB-E9 to the α-subunit of Na^+,K^+-ATPase partially inhibits enzyme activity (35%) in competition with ATP, while in the presence of magnesium it stimulates the rate of ouabain binding severalfold [Ball, W. J. (1984) *Biochemistry 23*, 2275–2281]. These effects have been shown to result from an antibody-induced shifting of the enzyme's $E_1 \leftrightarrow E_2$ conformational equilibrium to the right that affects all enzyme–ligand interactions except that with Mg^{2+} [Abbott, A. J., & Ball, W. J. (1992) *Biochemistry 31*, 11236–11243]. In order to identify the location of the M7-PB-E9 epitope, proteolytic fragments of the lamb kidney enzyme were generated and the immunoreactive α fragments were identified by Western blot analyses. These studies revealed a 47-kDa tryptic fragment, which bound both M7-PB-E9 and a -COOH terminus specific antisera and NH_2-terminal sequencing showed to originate at Ala-590. Digestion with *Staphylococcus aureus* V8 protease produced a 36-kDa -COOH-terminus fragment which originated at Gly-697 and did not contain the antibody epitope. Thus the intracellular sequence region Ala-590 to Gly-697 was shown to contain the antibody epitope. When M7-PB-E9's ability to recognize the α subunits from various species and tissues was determined and correlated with available sequencing data, only Ser-646 was present in the highly reactive lamb, pig, and avian kidney α1 proteins and altered (Asn) in the poorly recognized *Xenopus* and rat kidney and *Torpedo* electroplax organ enzymes. In addition, M7-PB-E9 was found to have a high binding affinity for the rat α3 isoform and low affinity for α2 which is altered at Asp-652 → Glu. The M7-PB-E9 epitope therefore was found to encompass the 7 amino acid sequence from Ser-636 to Asp-652. This site plays an important role in the $E_1 \leftrightarrow E_2$ conformational transitions undergone by the enzyme and it lies within a unique domain that links the highly conserved nucleotide binding and hinge domains of α as proposed by MacLennan et al. [(1985) *Nature 316*, 696] for the homologous sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase.

Na^+,K^+-ATPase (EC 3.6.1.37) is the integral membrane protein which actively pumps Na^+ and K^+ across the plasma membrane of animal cells, enabling them to maintain or restore their electrochemical gradients using the energy of ATP hydrolysis [see Jørgensen (1986)]. Pharmacologically, this enzyme is important since it serves as the specific receptor for cardiac glycosides (Schwartz et al., 1982). The enzyme consists of an ~110-kDa catalytic, or α-subunit, and an ~55-kDa glycosylated β-subunit. The α-subunit forms a phosphorylated intermediate and possesses the active sites for all known ligands and inhibitors of the enzyme [see review by Jørgensen (1988)]. In contrast, the β-subunit has no known enzymatic function but is apparently necessary for proper insertion of the α-subunit into the plasma membrane (Geering, 1991).

Knowledge of the Na^+,K^+-ATPase structure and its relation to enzyme function has recently been advanced by a combination of several techniques. The amino acid sequence for α1 (and the isoforms, α2 and α3) from a variety of species and tissues has been deduced from cDNA clones [reviewed in Lingrel et al. (1990)]. These sequence data have shown that α contains 11 hydrophobic regions of which 8 (H1–H8) were originally proposed to be transmembrane segments (Shull et al., 1985). Although the exact number of transmembrane segments is still uncertain [see Ovchinnikov et al. (1987), Bayer (1990), Antolovic et al. (1991)], within this general topographical arrangement several functional regions have been identified. For example, reactive ATP analogs whose binding is blocked by ATP covalently label α at Cys-656 and Lys-719 (Ohta et al., 1986), Asp-710 (Ovchinnikov et al., 1987), and Lys-480 (Hinz & Kirley, 1990), while ATP phosphorylation is at Asp-369 (Bastide et al., 1973). The fact that all of these sites are between the proposed H4 and H5 transmembrane sequences is consistent with this intracellular segment representing the ATP binding and hydrolyzing site. In addition, Glu-31, Ala-267, and Arg-438 have been identified as conformationally sensitive proteolytic cleavage sites (Jørgensen & Collins, 1986) which reside in the NH_2 terminus and the H2-H3 and the H4-H5 cytoplasmic domains, respectively. Cardiac glycoside binding which occurs on the extracellular side of the membrane has also been investigated by site-directed mutagenesis (Price & Lingrel, 1988) and found to be highly sensitive to changes in the amino acids (Gln-111 and Asp-122) which are located on the borders of the H1-H2 extracellular domain. Together, all of these identified sites define protein sequence regions involved in ligand binding or conformational changes, but there are still large regions of the protein structure whose function is undefined.

The correlation of amino acid sequences with enzyme function can also be achieved by raising antibodies that affect enzyme function by binding specific epitopes. This technique can be used to define amino acid (aa) sequences that are important for the enzyme's catalytic mechanism but which are not part of a ligand binding site or do not possess residue groups suitable for chemical labeling. Thus far, this approach

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has been only partially successful since antibodies that affect Na^+,K^+-ATPase function (Ball, 1984, 1986; Satoh et al., 1989; Schenk et al., 1984) have by-in-large displayed conformation-sensitive binding and therefore have proven difficult to map while antibodies directed against specific, known epitopes (Ball & Loftice, 1987; Antolovic et al., 1991; Felsenfeld & Sweadner, 1988) have generally had little or no effect on enzyme function.

In this work we have begun the identification of functionally important epitopes using the antibody M7-PB-E9, which has been shown to increase the enzyme's affinity for K^+, P_i, and ouabain by enhancing the $E_1 \rightarrow E_2$ conformational transition (Ball, 1984; Abbott & Ball, 1992). Because this antibody binds native and denatured enzyme, it has proven suitable for epitope mapping by Western blot analysis of intact and proteolytic α fragments. We have found that the epitope location resides in the H4/H5 cytoplasmic region of α which is adjacent to the putative nucleotide binding domain within an amino acid sequence domain that has not previously been associated with any specific function.

MATERIALS AND METHODS

Enzyme Isolation. Na^+,K^+-ATPase was purified from lamb and dog kidney by the method of Lane et al. (1979), and these preparations had initial specific activities of 1000 and 400 μmol of P_i/(mg·h), respectively. The pig kidney enzyme was purified by the method of Jørgensen (1974) and was a kind gift from Dr. H. Ping Ting-Beall (Mechanical Engineering and Materials Science, Duke University, Durham, NC) with an activity of 800 μmol of P_i/(mg·h). Microsomal preparations from chicken and *Xenopus* kidney, rat brain, and skeletal muscle and *Torpedo* electroplax organs were prepared by differential centrifugation of tissue homogenates. Frozen tissues, thawed, and then homogenized in 0.03 M imidazole, 2 mM EDTA, 0.25 M sucrose, pH 7.2, were centrifuged 10000*g* for 45 min, and the supernatant microsomes collected by centrifugation at 30000*g*, for 45 min. Microsome activities were between 30 and 100 μmol of P_i/(mg·h) except the *Torpedo* electroplax sample which was 0.6 μmol of P_i/(mg·h).

Trypsin Digestion and Fractionation of Peptides. Purified lamb kidney Na^+,K^+-ATPase (1–2 mg/mL) in 0.1 M Tris, 2 mM EDTA, 20 mM KCl, pH 8.0, was incubated for various times with a $^1/_{10}$ or $^1/_{20}$ weight equivalent of TPCK-trypsin (Worthington, Freehold, NJ). Proteolysis was stopped by addition of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (5% SDS, 0.1% β-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 6.9), heating 5 min at 60 °C, and the immediate loading of the samples on gels for electrophoresis. Slab gel electrophoresis was performed as described by Laemmli (1970), using a 10% acrylamide running gel and 5.5% stacking gel.

Western Blot Analysis. The SDS–PAGE resolved peptides were transferred from slab gels to nitrocellulose or poly(vinylidene difluoride) (PVDF) (Bio-Rad, 0.2 μm) solid membrane supports by electrophoresis for 2 h at 200 mA. Strips from these sheets were then incubated for 2 h with the primary monoclonal or polyclonal antisera diluted to 10 μg/mL or a 1/75 dilution, respectively, into buffer B (3% goat serum, 3% BSA, 0.05% Tween 20, 50 mM Tris, 200 mM NaCl, pH 7.4). The strips were then washed with buffer B followed by incubation with the appropriate horseradish peroxidase conjugated goat anti-mouse antibodies or goat anti-rabbit antibodies for 1 h and then washed again with buffer B and developed using the substrates H_2O_2 and 4-chloro-1-naphthol.

Peptide NH_2-Terminal Sequence Analysis. Na^+,K^+-ATPase tryptic peptides transferred to PVDF membranes were identified by staining with 0.075% Coomassie blue (1 min) and with M7-PB-E9 antibody by Western blotting. The dye-stained peptide bands were sequenced using an Applied Biosystems 470a gas phase sequenator, and the generated phenylthiohydantoin derivatives were analyzed on a Waters Nova-PTH C_{18} reverse-phase column. All peptide sequencing was performed by the Protein Chemistry Core Facility of the Department of Pharmacology and Cell Biophysics of the University of Cincinnati College of Medicine.

V8 Protease Digestion and Fractionation of Peptides. Purified sheep kidney Na^+,K^+-ATPase was also partially digested by *Staphylococcus aureus* V8 endoproteinase glu-C (V8 protease; Boehringer Mannheim, Indianapolis, IN). Purified lamb kidney Na^+,K^+-ATPase (250 μg) in 0.1 M Tris-buffered phosphate, pH 7.8, was digested for 1 h with 8.5 μg of V8 protease at 37 °C and then 8.5 μg more of the protease was added for overnight. The reaction was stopped by SDS–PAGE as described for the trypsin digestion. Subsequent electrophoretic transfer was to PVDF membranes, while Western blotting and NH_2-terminal amino acid sequencing were accomplished as described previously.

Materials. Buffers, ion salts, and enzyme substrates were from Sigma (St. Louis, MO). Antibodies conjugated to β-galactosidase and the horseradish peroxidase were from Gibco-BRL (Gaithersburg, MD) and Calbiochem (San Diego, CA), respectively, and electrophoretic supplies were from Bio-Rad (Richmond, CA).

RESULTS

Monoclonal antibody M7-PB-E9 raised against the sheep kidney Na^+,K^+-ATPase partially inhibits ATPase activity but stimulates the rate of ouabain binding (Ball, 1984) by enhancing ligand induced $E_1 \rightarrow E_2$ transitions and reduces enzyme affinity for Na^+ and ATP (Abbott & Ball, 1992). This antibody binds both native lamb kidney holoenzyme and the Western blotted lamb $\alpha 1$ but not the rat kidney α_1 isoform (Ball & Lane, 1986). These results suggested that analysis of its binding to Western blotted proteolytic fragments of the lamb enzyme and to the intact α from a variety of different species could be correlated with specific amino acids and its epitope identified.

Identification of Tryptic Fragment Containing the M7-PB-E9 Epitope. In order to identify which major domain of α contained the epitope, the holoenzyme was digested with trypsin in the presence of K^+ for various time periods and then fractionated by SDS–PAGE, transferred electrophoretically to nitrocellulose, and identified by immunoblotting (see Materials and Methods). In addition to mapping the α fragments recognized by M7-PB-E9, polyclonal antisera directed against the lamb $\alpha 1$ NH_2 terminus (amino acids 16–30), sequence position 823–833 and the -COOH terminus (amino acids 1003–1013), generated by using synthetic peptide -KLH conjugates as antigens (Ball & Loftice, 1987), were used to identify proteolytic fragments containing these specific sequence regions. After establishing the recognition of intact α by the three antisera and M7-PB-E9 (Figure 1, groups A and E), the initial exposure of the enzyme to trypsin ($\sim^1/_{20}$ by weight, Figure 1, group D) for 1.5 min essentially generated the NH_2-terminal, 41-kDa and -COOH-terminal 58-kDa (actually a doublet) fragments reported previously by Jørgensen and Collins (1986). Ten minutes of trypsin digestion ($\sim^1/_{10}$ by weight, Figure 1, group B) then reduced the 41-kDa NH_2-terminal fragment to a 35-kDa fragment, while M7-PB-E9, and the two -COOH-terminal region directed sera recognized a similar set of five major fragments ($\sim$77, 58, 42, and 37 kDa). At 60 min (Figure 1, group C), except

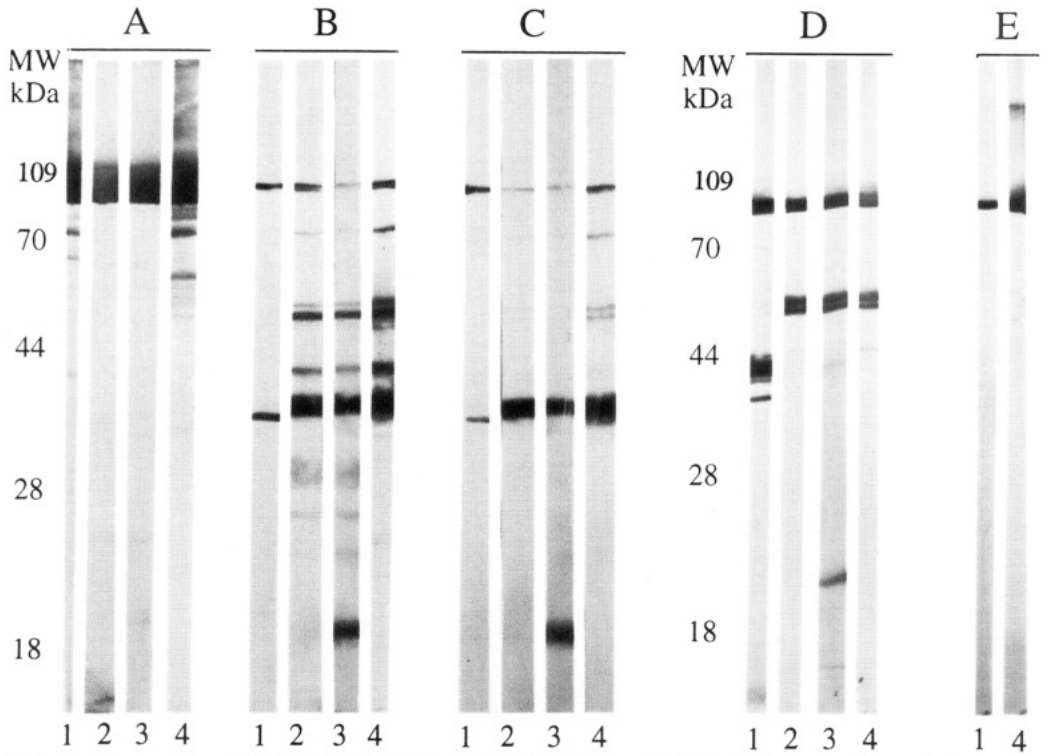

FIGURE 1: Trypsin fragment mapping of the M7-PB-E9 binding site. Sheep kidney Na^+,K^+-ATPase was digested with trypsin (at 1/10 ratio, w/w) for 0 min (strips of groups A and E), 10 min (group B), or 60 min (group C) and in a separate experiment (trypsin 1/20, w/w) for 1.5 min (group D). Digest samples were resolved by SDS–PAGE, in 10% gels, and then electroblotted onto nitrocellulose paper, and strips representing each digest sample were stained using the different antibodies. The nitrocellulose strips in each lettered group were exposed to the following antisera or antibody: strip 1, an antiserum directed against the NH_2-terminus lamb α1 sequence peptide of aa 16–30; strip 2, an antiserum to the internal sequence, 823–833; strip 3, an antiserum to the -COOH-terminal sequences, 1000–1013; strip 4, the monoclonal antibody, M7-PB-E9; samples in groups A, B, C and D contained 80 μg of Na^+,K^+-ATPase while sample E was 8 μg.

for one 20-kDa fragment which contained the -COOH terminus, a ~37-kDa α fragment which retained its reactivity with the 823–833, and -COOH-terminus specific antisera and M7-PBE9, and a ~35-kDa NH_2-terminus serum reactive fragment predominated. When the enzyme was digested for longer than 60 min, the intensity of the 35- and 37-kDa bands diminished somewhat and only for the -COOH terminus binding serum were any strongly immunoreactive peptide fragments of lower molecular weight generated. This presumably was because any additional digestion destroyed the epitopes or the antibody staining patterns became excessively diffuse when the peptides were in the lower molecular weight range. These results showed that the epitope for M7-PB-E9 resides on the -COOH terminal portion of α.

It should be pointed out that the undigested holoenzyme showed aggregation and trace levels of α fragments (Figure 1, group A) when blotted at the concentrations needed for the proteolysis experiment. None of these bands, however, matched those observed in the trypsin digests (Figure 1, groups B, C, and D), and lower enzyme concentrations (Figure 1, group E) eliminated these overloading effects.

In order to identify the smallest proteolytic fragment containing the M7-PB-E9 epitope, the 37-kDa tryptic fragment was isolated and sequenced at its NH_2 terminus. In this experiment, the holoenzyme was trypsin digested 60 min and the resulting peptides were fractionated using SDS–PAGE and electrophoretically transferred to a (PVDF) membrane. Figure 2 shows the protein distribution as identified both by Coomassie blue staining and by Western blotting of the same sample with M7-PB-E9. In addition to intact α at ~110-kDa, major protein bands were observed at ~58, ~37, and 35 kDa. Antibody M7-PB-E9 recognized the 110-, 58-, and 37-kDa bands but not the 35-kDa protein band. An additional band at ~44 kDa could be identified clearly by M7-PB-E9 but poorly by the less sensitive Coomassie blue staining. The 37-kDa band was therefore cut from the PVDF sheet and sequenced by automated Edman degradation (see Materials and Methods). A single amino acid sequence, with a 21–23 pmol yield, was found in each of the first three cycles of the gas-phase amino acid sequenator. As shown in Figure 2, the first eleven amino acids detected aligned with the lamb kidney α1 sequence starting at Ala-590. Thus, the M7-PB-E9 epitope resides on an apparent 37-kDa fragment (~47 kDa for the calculated molecular mass) of α whose NH_2 terminus is Ala-590 which also retains the 823–833 and the -COOH-terminal (1003–1016) sequences.

Species-Specific Binding of M7-PB-E9. Monoclonal antibody M7-PB-E9 binds the sheep kidney α1 but not rat kidney α1 in both ELISA and Western blot assays (Ball & Lane, 1986). Because both proteins are highly denatured in the Western blot analysis, it is likely that amino acid sequence rather than conformational differences in the two proteins caused this difference in antibody binding. Analysis of the two α sequences showed that there are only 11 amino acid differences between these proteins in the Ala-590→-COOH-terminal fragment that contains the M7-PB-E9 epitope. In order to more specificaily locate the M7-PB-E9 epitope, the antibody binding to microsomes or isolated enzyme from lamb, pig, dog, chicken, rat, and *Xenopus* kidney and *Torpedo* electroplax tissues was surveyed using Western blot analysis of gel-resolved α proteins. Figure 3 shows that the control polyclonal antisera monospecific for the Na^+,K^+-ATPase α-subunit recognized the α protein in all tissue samples. Monoclonal antibody M7-PB-E9, however, stained the lamb, pig, and chicken kidneys and rat brain but did not stain rat and *Xenopus* kidney or *Torpedo electroplax* α. However, when this experiment was repeated with high amounts of antigen (i.e., 100 μg of microsomes per gel lane), M7-PB-E9 faintly stained the α bands of the unreactive tissues (data not shown). M7-PB-E9 also reacted strongly with the dog kidney

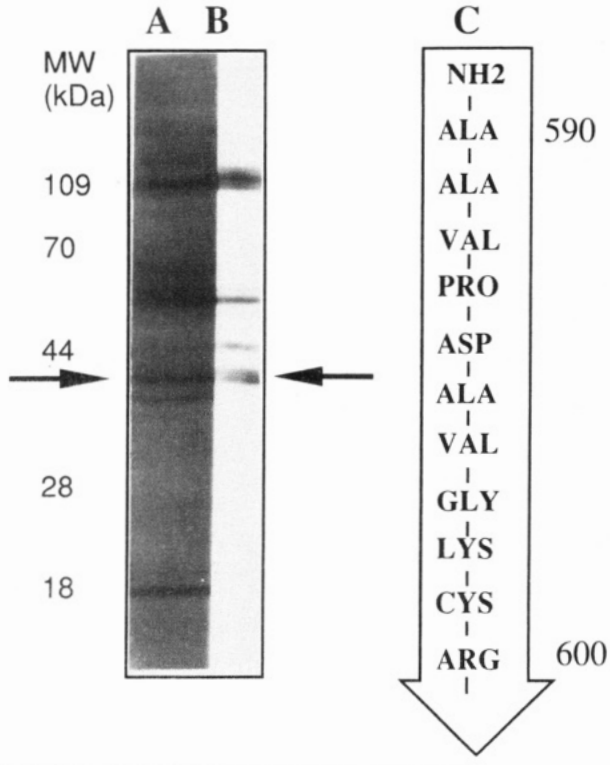

FIGURE 2: NH_2-terminal sequencing of the M7-PB-E9 epitope containing peptide. Sheep kidney Na^+,K^+-ATPase (400 μg) was partially digested with trypsin (40 μg) for 60 min and electroblotted from a slab gel as in Figure 2, onto a PVDF membrane. The lowest molecular weight peptide band (37 kDa by gel migration, or 46 kDa by sequencing) that was stained by both Coomassie blue dye (lane A) and by mAb M7-PB-E9 (lane B; the lower band in doublet marked by arrow) was sequenced from the NH_2-terminus end (lane C). The resulting 11 amino acid sequence was identical to the known α sequence beginning at Ala-590 and as shown in Figure 1; the peptide also contained the α subunit's -COOH terminus.

enzyme (data not shown), but this result was not used for any subsequent analysis, because while its sequence is similar to lamb it has not yet been fully reported.

An ambiguity in the results shown in Figure 3 resulted when M7-PB-E9 was observed to bind rat brain but not rat kidney samples. Since the brain tissue is known to contain all three isozymes (α1, α2, and α3) of Na^+,K^+-ATPase (Shull et al., 1986) while the kidney contains only α1, it appeared that one or both of the rat α2 and α3 isozymes were recognized by the antibody. To determine the reactive isozyme(s), Western blot analysis of M7-PB-E9 binding to rat skeletal muscle miscrosomes as well as brain and kidney was done because skeletal muscle contains predominantly α2. Also, in order to ensure that similar amounts of enzyme were being assayed, samples containing equivalent amounts of ouabain inhibitable Na^+,K^+-ATPase activity rather than protein were analyzed for antibody binding to these tissue membranes. Figure 4 shows that enzyme equivalent to 0.23 μmol of P_i/h (roughly equivalent to 0.23 μg of purified Na^+,K^+-ATPase) from freshly prepared microsomes was clearly detectable by M7-PB-E9 in brain tissue ($\alpha1 + \alpha2 + \alpha3$) but not in skeletal muscle (α2) or in kidney (α1). This revealed a binding specificity of M7-PB-E9 for the rat α3 isozyme.

The species and isozyme specificity of M7-PB-E9 binding was then analyzed in terms of the known α sequences in order to identify the antibody's epitope (see Figure 5). The reactive α1 of the lamb kidney enzyme (sequences Ala-590 → -COOH terminal) differs from rat α1 at 11 amino acids, located in five general sequence regions. Inspection of these regions revealed that only Ser-646 of lamb α1 was conserved in all reactive species and altered to Asn in three of the four nonreactive species (i.e., rat and *Xenopus* kidney and *Torpedo electroplax*). The remaining unreactive tissue, rat skeletal muscle (α2 isozyme), is unchanged at Ser-646 but contains a substitution of Asp-652 → Glu. These data suggest that these two amino acids (Ser-646 and Asp-652) are critical for antibody binding and that the M7-PB-E9 epitope encompasses this 7 amino acid stretch of the lamb kidney α protein. The epitope should not extend beyond Ala-653 because the lamb α1 is uniquely altered at Arg-654 with all other α's having a Lys at this site. We should also point out that M7-PB-E9 showed no reactivity with the rabbit skeletal sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase (data not shown) and that there is no sequence homology between the Na^+ and Ca^{2+} pumps within this stretch of sequence.

Identification of the M7-PB-E9 Epitope and -COOH-Terminal Sequence in V8 Protease Fragments of α. In order to obtain a smaller or different peptide containing the antibody epitope that would provide additional information about the M7-PB-E9 site, the enzyme was digested with V8 *aureus* protease. The resulting peptides were fractionated by SDS–PAGE, transferred to a PVDF solid support, and identified by both Coomassie blue staining and antibody staining as previously presented. Figure 6 shows that V8 protease digestion generated fragments quite distinct from those produced by trypsin. While the -COOH terminus directed antisera (Figure 6, lane 2) and M7-PB-E9 (lane 3) both bound 70-kDa and 44-kDa fragments, in general they bound different fragments. Further, unlike in the trypsin digestion, M7-PB-E9 bound a number of fragments smaller than 30 kDa, with an 18- and 16-kDa fragment being especially reactive. These latter two fragments, however, were not particularly evident from direct protein staining. Therefore, in this experiment the ~27-kDa fragment which stained well with Coomassie blue and the -COOH terminus antisera, but not with M7-PB-E9, was excised and sequenced. This region of the PVDF paper showed a major sequence (20 pmol/residue) from α and a minor sequence (5 pmol/residue) from the β-subunit. The α peptide started at Gly-697 as indicated by the seven-residue sequence Gly-Cys-Gln-Arg-Gln-Gly-Ala. Therefore, the M7-PB-E9 epitope is excluded from the α sequence region of Gly-697 to Tyr-1016 of the -COOH terminus. The β-peptide started at Ile-87 and it was detected by Western blot analysis using an anti-β subunit directed serum (Figure 6, lane 4). The presence of the M7-PB-E9 epitope within the α sequence from Ala-590, including 823–833 to the -COOH terminus and its exclusion from Gly-697 and beyond limits its location to the 106 amino acid, Ala-590 to Glu-696 region. This is entirely consistent with the species specificity mapping results which identified Ser-646 and Asp-652 as critical for antibody binding.

DISCUSSION

In this report, the epitope for monoclonal antibody M7-PB-E9 was identified to include the Ser-646 → Asp-652 sequence of the sheep kidney Na^+,K^+-ATPase which lies within a protein sequence that has, thus far, not been ascribed any specific function. The sequence domains flanking the epitope, however, have been assigned to be the nucleotide binding and hinge domains as based upon the currently available experimental and molecular modeling evidence (see below). Because our recent functional studies have shown that antibody M7-PB-E9 alters the enzyme's ligand-induced $E_1 \rightleftarrows E_2$ conformational transitions and thereby affects ATP, ouabain, and cation binding (Abbott & Ball, 1992), we hypothesize that the epitope resides in a functionally important sequence region that allosterically links both intra- and extramembranous domains of α1.

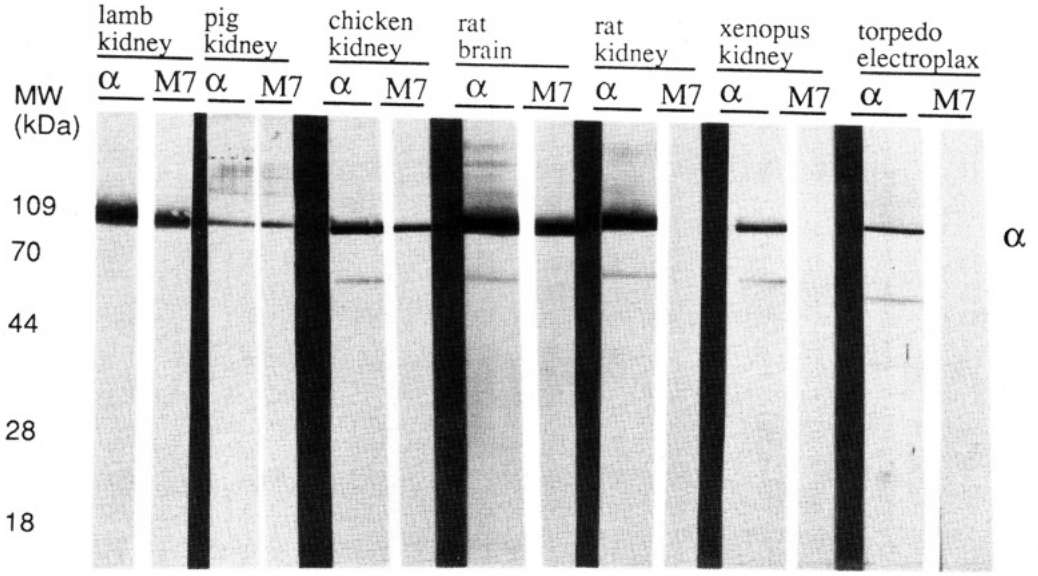

FIGURE 3: Species and tissue specific binding of M7-PB-E9 to Na^+,K^+-ATPase. Purified enzyme (1 μg) or microsomal preparations (20 μg) were SDS–PAGE resolved and Western blot analyzed using an anti-lamb kidney α-subunit directed rabbit serum (designated as α) and mAb M7-PB-E9 (designated as M7). While all tissues were recognized by the anti-α-subunit polyclonal antibodies, lamb, pig, and chicken kidney and the rat brain enzymes bound M7-PB-E9 while rat and *Xenopus* kidney and *Torpedo electroplax* organ enzyme did not.

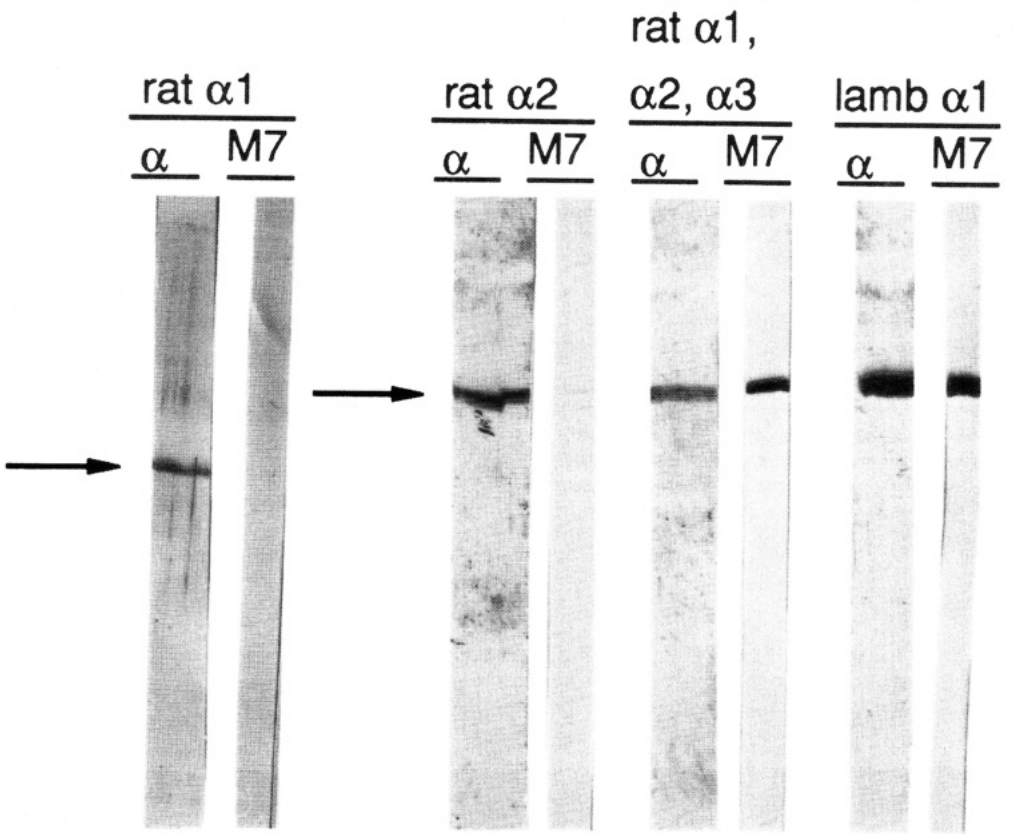

FIGURE 4: Determination of M7-PB-E9's specifity for rat Na^+,K^+-ATPase isozymes. M7-PB-E9 binding to Western blotted Na^+,K^+-ATPase isozymes was compared for rat kidney (rat α_1), rat skeletal muscle (rat α_2), rat brain (rat α_1, α_2, α_2), and lamb kidney (lamb α_1). All lanes contained matched levels of enzyme activity rather than protein (0.23 μmol of P_i/(mg·h) or about 0.23 μg of enzyme), and duplicate lanes were exposed to the α-subunit specific antisera (α) and M7-PB-E9 (M7), respectively. Antibody M7-PB-E9 specifically bound rat α3 but not rat α1 or rat α2. The arrows mark the positions of the α subunit (~110 kDa) as identified by comparison with Coomassie blue stained protein standards. Although not shown, these rat kidney and skeletal muscle samples showed strong reactivity with the appropriate isoform specific monoclonal antibodies McK1 (α1) and McB2 (α2) generously supplied by Dr. Kathleen Sweadner (Harvard Medical School).

The determination that the epitope must contain Ser-646 and Asp-652 of the lamb α1 is based on two lines of evidence: The first was established by correlating known α amino acid sequences with the antibody's ability to bind the enzyme from several species and specific isozyme containing tissues. The two identified amino acids lie within a peptide sequence small enough to be a continuous epitope of 7 amino acids and they are the only such amino acids consistently conserved in antibody reactive proteins but altered in unreactive enzymes. The second approach was to isolate and identify proteolytic α fragments that bind the antibody. The putative epitope was found to lie within an 11-kDa sequence region of the lamb kidney enzyme (Ala-590 to Glu-696) as defined by the antibody's reactivity with the tryptic peptide Ala-590 → Tyr-1016 and its lack of reactivity with the *S. aureus* V8 protease generated peptide Gly-697 → Tyr-1016.

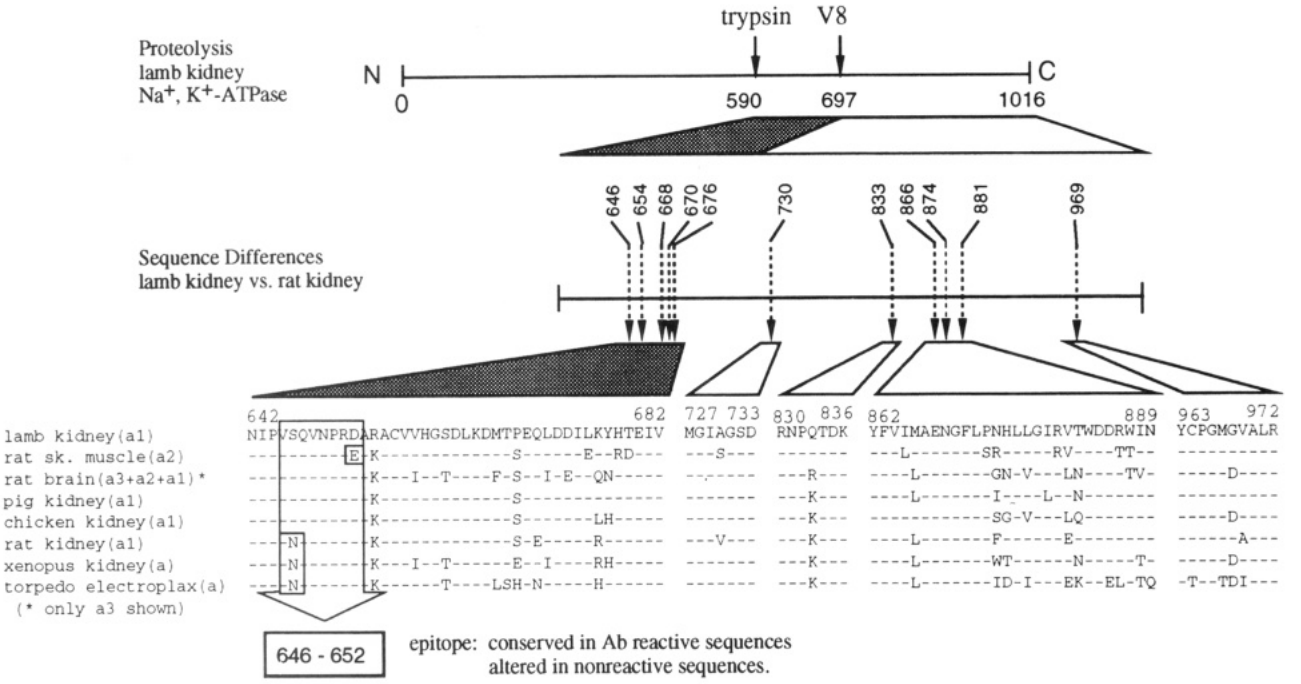

FIGURE 5: Analysis of the evidence that the M7-PB-E9 epitope contains Ser-646 and Asp-652. Trypsin digestion of the lamb kidney enzyme revealed that the Ala-590 to Tyr-1016 α peptide contained the M7-PB-E9 binding site (Figures 2 and 3). Its sequence, however, differs from that of the unreactive rat α_1 at only 11 amino acid residue sites. This defined five potential epitope sequence regions, but only two residues (Ser-646 and Asp-652) were defined by matching sequence identities for antibody reactive tissue and sequence mismatches for the four unreactive tissues: rat and *Xenopus* kidney, *Torpedo electroplax*, and rat skeletal muscle α2. The shaded portion of the sequence map shows the epitope region defined by trypsin and V8 protease digestion (see Figure 6).

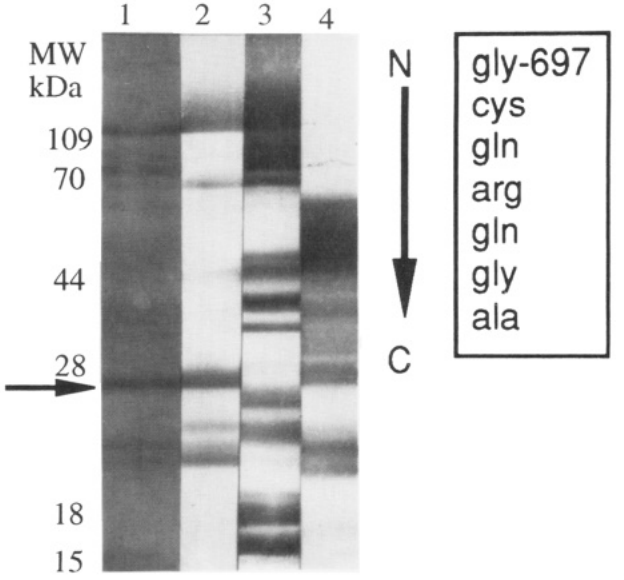

FIGURE 6: NH_2-Terminal sequencing of a ~27-kDa peptide containing the enzyme's -COOH terminus but lacking the M7-PB-E9 epitope. Sheep kidney Na^+,K^+-ATPase (250 μg) was digested with V8 protease (see Materials and Methods), and the mixture was resolved and then electroblotted onto PVDF membrane as described in Figure 2. The arrow identifies a predominant peptide band that could be identified by Coomassie blue staining (lane 1) and by a -COOH-terminus directed antisera (lane 2) but not by M7-PB-E9 (lane 3). The NH_2 terminus of this fragment was found to begin at Gly-697 of α. Lane 4 shows the digestion fragments of β produced as detected by a β-directed antiserum.

We should point out that in an earlier study Farley et al. (1986) reported some initial mapping work done with M7-PB-E9 and suggested that it bound to both portions of a discontinuous epitope or a repeated sequence contained in an NH_2-terminal 41-kDa fragment and a nonoverlapping -COOH-terminal 58-kDa fragment. Our tryptic fragmentation work gave essentially the same fragment binding pattern (~58, 42, and 37 kDa) that he obtained with the dog enzyme. However, without NH_2- and -COOH-terminal specific markers such as our synthetic peptide-directed antibodies he was not able to differentiate between similar molecular weight fragments originating from different regions of the polypeptide.

Although several monoclonal antibodies have been reported that significantly alter Na^+,K^+-ATPase function (Ball, 1984, 1986; Schenk et al., 1984; Satoh et al., 1989; Urayama et al., 1990), none of these epitopes have been identified. Recently, however, an approach similar to ours has been used to map the epitope for the H^+,K^+-ATPase-directed inhibitory antibody, 5-B6 (Van Uem et al., 1991) and for the Na^+,K^+-ATPase-directed antibody VG_4 (Arystarkhova et al., 1992). Other studies have defined antibody epitopes by using short synthetic peptides and their conjugates as either polyclonal antibody immunogens or as test antigens for the screening of monoclonal antibody in order to identify epitope-containing peptides. These antibodies, however, have had little or no effect on enzyme activity (Ball & Friedman, 1987; Antolovic, 1991).

The M7-PB-E9 epitope location, based on our evidence and Farley et al.'s (1986) evidence, is on a cytoplasmically exposed region between the enzyme's fourth and fifth transmembrane segments (H4-H5, Lys-342 to Glu-779) that contains the ATP binding and phosphorylation sites. Near its NH_2-terminal (H4) end, Asp-369 is phosphorylated by ATP (Bastide et al., 1973) and at Lys-480 begins a sequence region that reacts with the ATP analogs, pyridoxal 5′-diphospho-5′-adenosine (AP_2PL) (Hinz & Kirley, 1990), 5′-(*p*-fluorosulfonyl)benzoyladenosine (5′-FSBA) (Ohta et al., 1986), and γ-[4-(*N*-(2-chloroethyl)-*N*-methylamino)]benzylamide-ATP (C1RATP) (Ovchinnikov et al., 1987), which have been shown to label Lys-480, Cys-656 and Lys-719, and Asp-710 and Asp-714, respectively. In addition, fluorescein 5′-isothiocyanate (FITC) labeling of Lys-501 completely inhibits ATPase activity and its binding is competitive with ATP (Kirley et al., 1984; Farley et al., 1984).

Although the nucleotide bases of the ATP analogs are likely to bind in the ATP binding site, their reactive groups (excluding FITC) are on the adenosine structure in a position that appears to mimic ATP's terminal phosphate. This is important because many ATP hydrolyzing enzymes have separate ATP binding and phosphorylation domains (Bennet & Huber, 1984), either of which could be labeled by an analogs' reactive groups. Exactly which amino acid sequences on Na^+,K^+-ATPase constitute these domains is unclear, except that Asp-369 is the phosphorylation site and this region must be part of the phosphorylation domain. Therefore, the M7-PB-E9 epitope, Ser-646 to Asp-652, could spatially reside near either of the two domains associated with ATP binding and hydrolysis.

Our prediction is that the antibody's epitope is not part of either the ATP binding or phosphorylation domains. This is based not only on our functional studies but also on amino acid sequence comparisons between Na^+,K^+-ATPase and the Ca^{2+},Mg^{2+}-dependent ATPase from rabbit skeletal muscle sarcoplasmic reticulum (SR-Ca^{2+}-ATPase). We assume that their ATP binding sites have similar structures. In support of this assumption, both enzymes have similar high ATP binding affinities (K_d of about 1–5 μM; Dupont et al., 1985; Moczydlowski & Fortes, 1981) under analogous conditions, and phosphorylation occurs at the same homologous site. Both are also labeled, by the discussed ATP analogs, at similar points in the protein (Bastide et al., 1973; Allen & Green, 1976). Overall, the Na^+,K^+-ATPase sequence region that includes all ATP affinity labeling sites, Lys-480 → Asp719, is 41% identical to SR-Ca^{2+}-ATPase (Shull et al., 1985; MacLennan et al., 1985).

By comparison, the 57 amino acid sequence domain (Ile-627 → Val-682) of Na^+,K^+-ATPase which contains the M7-PB-E9 epitope has no sequence identity to the matching SR-Ca^{2+}-ATPase region [see Shull et al. (1985)]. Interestingly, the length of the nonidentical sequence is only 35 amino acids in SR-Ca^{2+}-ATPase, and this difference (57 vs 35 amino acids) indicates that DNA deletions and/or insertions rather than point mutations created this divergence in these sequences. Because these nonhomologous sequences are in enzymes with similar structure and function (see above), they are not likely to be part of the respective ATP binding or phosphorylation sites.

There is other information on the phosphorylation and ATP binding sites of E_1E_2-type ATPases that can be used to predict the function of the sequences that border the -COOH side of the M7-PB-E9 binding domain. For example, Vilsen et al. (1991) found that mutations of amino acids Lys-684, Asp-703, and AsnAsp-707 on the expressed SR-Ca^{2+}-ATPase eliminated phosphoenzyme intermediate formation but not ATP binding. These amino acids are in the so-called "hinge" region of the enzyme (aa 675–715, in the Ca^{2+}MgATP; MacLennan, 1990) which is highly conserved among E_1E_2 ATPases (61% identity, with aa 683–723, of Na^+,K^+-ATPase) and they reside on the -COOH-terminal side of the M7-PB-E9 binding domain beginning only 10 amino acids downstream. While the primary sequence of this hinge region is quite distant from the phosphorylation site, it appears to physically reside close to Asp-367 and be intimately involved in the phosphorylation mechanism. For the nucleotide binding site, Taylor and Green (1989) have predicted the consensus nucleotide binding site sequences for the E_1E_2-type ATPases by comparing their predicted secondary structural similarities to the nucleotide binding domains of enzymes with known structures including adenylate kinase, phosphofructose kinase, and F_1-ATPase. The most likely sequences were found to be the Ala-491 → Ile-626 segment which is contiguous with the

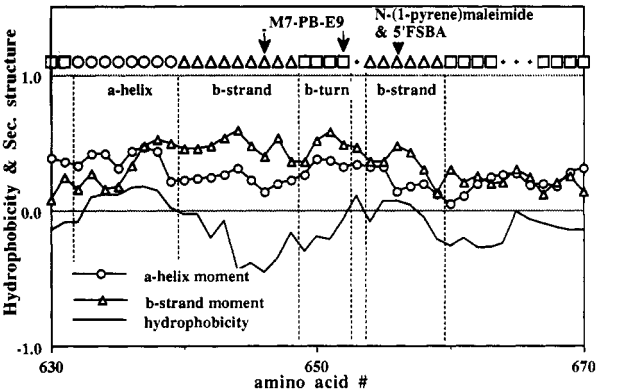

FIGURE 7: Secondary structural predictions at the M7-PB-E9 epitope. For the sequence region 630–670 which contains the M7-PB-E9 epitope (Ser-646 to Asp-652), the method of Chou and Fasman was used to predict α-helix (circles), β-sheet (triangles), β-turn (squares), or unpredicted (+) 2° structures for the α subunit. In addition, both the average hydrophobicity (solid line) and hydrophobic moments for α-helix (circles) and β-strand (triangles) were calculated using an averaging window of 9 amino acids. All calculations were performed on an Apple IIC personal computer using the spreadsheet program EXCEL (Microsoft Corp.).

NH_2-terminal side of the M7-PB-E9 binding domain. The results from our studies all fit very well into this model.

This model is valuable in interpreting the relationship between the M7-PB-E9 epitope location and its effects on enzyme function, but it should be accepted with caution in view of our incomplete understanding of the enzyme's structure and it is contradicted by some evidence. For example, of the six previously mentioned sites labeled by ATP analogs, only one of them (Lys-501) is within the predicted nucleotide binding domain, but this is explained by having the five other sites as part of the phosphorylation domain. Also, the M7-PB-E9 epitope is adjacent to the Cys-656 labeled by the ATP analog 5'-FSBA. If this sequence is not functionally part of either the ATP binding or the hinge domain, then it must still be close enough to be labeled by this ATP analog. Nonetheless, the model is generally consistant with M7-PB-E9 binding at a region that affects enzyme conformational transitions and the interactions between the two discussed domains but does not directly alter ATP binding or phosphorylation. The enzyme's conformational transitions are known to be strictly coupled to ion translocation, but this is predicted to result from indirect interactions between the various subunit domains. Interestingly, Cys-656 is also one of two Cys (Cys-367 and Cys-656) whose labeling by *N*-(1-pyrene)maleimide is allosterically protected by ouabagenin binding (Kirley & Peng, 1991) while the analogous but nonidentical domain from SR-CA^{2+}-ATPase has been labeled by 5'-(iodoacetamido)-fluorescein (5'-IAF) and 5-(2-((iodoacetyl)amino)ethyl)-aminonaphthylene-1-sulfonic acid (IAEDENS) at Cys-670 and Cys-674 (Bishop et al., 1988), respectively, with no effect on enzyme function.

Secondary Structural Predictions. A striking feature of the M7-PB-E9 binding domain is its apparent propensity to form specific secondary structures. Using the Chou–Fasman method (Prevelige & Fasman, 1990), a specific secondary structure (i.e., α-helix or β-sheet or β-turn) is predicted when the conformational parameter (P) averaged over a particular amino acid stretch is greater than 1. Starting at Glu-632 → Gly-660, the predicted structural sequences are α-helix, followed by β-strand, β-turn, and β-strand, and they are highly favored with P values of 1.41, 1.19, 1.37, and 1.2, respectively, with no other 2° structures predicted. The remaining amino acids (660–682, but only to 670 is shown in Figure 7) are predicted to be either β-sheet or α-helix. The calculated

average hydrophobicity index and the hydrophobic moment (Eisenberg et al., 1984) are also shown for sequence groups of nine contiguous amino acids centered at the points shown in Figure 7. This analysis shows that this region does not have a strong hydrophobic character but does indicate a definite hydrophilic segment for Pro-644 → Asp-652, which includes the M7-PB-E9 binding site (Ser-646 → Asp-652). The hydrophobic moment then measures the periodicity in the hydrophobic index characteristic of α-helices (3.6 amino acid period) and β-sheets (2.3 amino acid period) on a scale from 0 to 1 and is a measure of the sequence's amphipathicity as induced by its 2° structure. The α-helix region identified (630–639) has a helix moment of 0.3–0.4, which suggests that it is likely to be surface exposed as based on the measurements of hydrophobicities and moments for known surface-exposed α-helix peptides (Eisenberg et al., 1984). The moment for the first β-strand (Arg-640 to Val-648) sequence is even higher (0.5–0.6) while the second β-strand (654–659) has a lower moment value (0.2–0.3). The first β-strand is therefore predicted to be amphipathic and solvent exposed and the second β-strand hydrophobic. As a working model, we can propose that Ser-646 of the antibody's epitope would then be on the polar side of this β-strand and Asp-652 on the following β-turn structure. Adjacent to these structures in the second β-strand is Cys-656 which is labeled by 5′-FSBA and *N*-(1-pyrene)maleimide. On the basis of the observation that the bound antibody does not affect eosin binding, we further propose that the first β-strand of the β-strand–β-turn–β-strand structure is solvent exposed, allowing the antibody to bind, while the second β-strand faces the ATP binding site, is buried in the protein, and is subject to chemical modification. This region should also be highly sensitive to its microenvironment. Since M7-PB-E9 binding alone does not appear to cause much distortion of the enzyme's conformation, it may be that bound antibody alters the hydrophilicity of the region such that the $E_1 \rightarrow E_2$ transition is facilitated. This appears consistent with the observation that the reactivity of Cys-656 with the hydrophobic reagent *N*-(1-pyrene)maleimide is allosterically dependent upon specific ligands as well as on the presence of ouabaginin (Kirley & Peng, 1991).

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