Fine Specificity Mapping and Topography of an Isozyme-specific Epitope of the Na,K-ATPase Catalytic Subunit*

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An isozyme-specific domain of the catalytic subunit of the Na,K-ATPase has been identified using a monoclonal antibody, McK1. The antibody's specificity was confirmed by its ability to stain proteolytic fingerprints of the Na,K-ATPase. The antibody recognized the α1 isozyme of the rat Na,K-ATPase, but not the αII or αIII isozymes (which are recognized native and sodium dodecyl sulfate-denatured Na,K-ATPase and specifically stained basolateral membranes of the renal tubule. It bound to rat α1 with highest affinity, but also cross-reacted with mouse, monkey, and human α1. It did not cross-react with sheep, pig, chicken, Torpedo, or dog α1. Fine specificity mapping was used to deduce the most likely antibody binding sites, based on comparison of eight amino acid sequences from cDNA clones. Two potential binding sites were found at widely separated locations. Limited tryptic digestion of the native enzyme was then used to demonstrate that the binding site(s) are located at the N-terminal end of the Na,K-ATPase. The binding site is predicted to include the following essential amino acid sequence: Asp-Lys-Lys-Asp-Lys-Gly-Lys-Lys in rat α1 or Asp-Lys-Lys-Gly-Lys in human α1. The antibody was found to bind to opened, not to sealed right-side-out vesicles isolated from the rat renal medulla, demonstrating that the N-terminal end of the Na,K-ATPase is exposed at the interior of the cell.

The Na,K-ATPase, which is responsible for the active transport of Na⁺ and K⁺ ions in animal cells, is a membrane-embedded protein (1). It contains two different protein subunits, α and β, both of which span the membrane bilayer. The α subunit is considered to be the catalytic subunit, because it contains the binding sites for ATP and ouabain while the function of the β subunit is still unknown. The structure of the Na,K-ATPase has been difficult to determine by direct biophysical approaches. The primary amino acid sequence is known, however, and predictions have been made about the location of several or eight possible transmembrane α-helices (3–7). Several sites of covalent modification have been determined and located in the polypeptide map, but many ambiguities remain, such as which amino acids contribute to the binding sites for ions and ouabain. In addition, it is not known what portions of the polypeptide are exposed to solvent and what portions are folded in the interior of the protein, and further evidence is needed to verify or disprove the location of the proposed transmembrane sequences. In principle, monoclonal antibodies could be used as probes in such studies of structure if their sites of binding were unambiguously known.

Three isozymes of the catalytic subunit of the Na,K-ATPase have been identified, two as proteins (8, 9) and a third as a cDNA and mRNA (7, 10–14). Multiple potential Na,K-ATPase genes have also been identified in the mouse and human genomes (15–17). Previous studies with rabbit antisera raised against purified rat kidney and axolemma Na,K-ATPases (18) indicated that the isozymes had distinct antigenic determinants. The sequences of the isozymes from cDNA clones indicate that they are derived from different genes, not from alternative splicing, and further that there are many potential sites for antigenic differences (10). In addition, there are now complete amino acid sequences for the α1-equivalent Na,K-ATPase catalytic subunits from six different species (3–5, 7, 10, 11, 19, 20).

The availability of a library of complete sequences makes it possible to analyze antigen structures by fine specificity mapping, which is the deduction of binding sites based on an amino acid-by-amino acid comparison of evolutionarily or mutationally related proteins. Fine specificity mapping has been employed extensively in the study of antigenic sites on to describe the isozyme with the N-terminal Met-Gly-Asp-Lys-Lys. The αI isozyme has a low affinity for ouabain in the rat; the αII isozyme (which we (8) and Lytton (9) originally called αII) has a high affinity; and the affinity of the αIII isozyme is still unknown. The form(s) of the enzyme that we have in the past called α(+) as a protein, on the basis of slower electrophoretic mobility in SDS (8), is likely to comprise both αII and αIII isoforms. We now use the term α(+) only when referring to an α-related protein of slower electrophoretic mobility when it reacts specifically with an antibody (A2) raised against the Na,K-ATPase purified from rat brainstem axolemma. When we use the term α(+) the molecular identity of the gene product (αII or αIII) is still not known.
proteins such as cytochrome c, lysozyme, myoglobin, and hemoglobin, where there are numerous observations of cases where single amino acid substitutions result in antigenic differences (21). When an antibody binds to denatured enzyme, and the juxtaposition of discontinuous portions of the linear polypeptide chain, fine specificity analysis can be used to deduce essential amino acids in the antibody binding site.

In this paper, we present an antibody that recognizes both native and denatured Na,K-ATPase and whose binding site has been studied by fine specificity mapping and peptide mapping. The antibody, McK1, defines a stretch of amino acids on the catalytic subunit that are exposed to the aqueous phase at the interior of the cell.

**Experimental Procedures**

**Hybridoma Production**—The antigen was Na,K-ATPase purified from the rat renal medulla by the procedure of Jorgensen (18, 22) and emulsified in complete Freund’s adjuvant. Several BALB/c mice were immunized intraperitoneally with 10 μg of enzyme at 0 weeks and again at 6 weeks; at 7 weeks, sera were drawn for evaluation of titer against kidney and axolemma Na,K-ATPase. One mouse was selected on the basis of a higher titer for kidney than for axolemma Na,K-ATPase, and it was boosted by tail vein injection of 40 μg of kidney Na,K-ATPase each day for 3 days. On the 4th day the spleen was removed and fused with myeloma cells. Supernatants from the wells were screened first for ability to bind to undenatured Na,K-ATPase in membranes from the rat renal medulla; about 80 positive wells were found. The positives were subsequently screened for their ability to bind to Na,K-ATPase on electrophoretic blots of SDS-polyacrylamide gels. Fourteen of the positive wells stained a band of the expected electrophoretic mobility for the α subunit of the Na,K-ATPase; McKl gave a particularly strong signal and was selected for subcloning and further analysis. After two rounds of subcloning, a mouse monoclonal isotyping kit obtained from HyClone Laboratories (Logan, UT) was used to determine the heavy chain subclasses.

**Gel Electrophoresis and Immunoblotting**—Gel electrophoresis and immunoblotting were performed as described previously (8, 18). The electrophoresis system of Laemmli was used (24), with recrystallized SDS and without heating of the samples. Proteins were blotted onto nitrocellulose by the procedure of Burnette (25), and the blots were stained with 0.5%-TWEEN 20 in Tris- or phosphate-buffered saline. The monoclonal antibody McK1 was the result of fusing a mouse myeloma cell line with cells from the spleen of a mouse immunized with the purified, membrane-associated Na,K-ATPase (α1 and β) isolated from the rat renal medulla. The hybridoma-containing wells were screened first for binding to undenatured Na,K-ATPase and second for binding to its subunits, separated by electrophoresis in SDS and blotted onto nitrocellulose. The antibody is of the heavy chain subclass IgG1.

Membrane preparations from rat axolemma, renal medulla, and brain (8) and from newborn cardiac ventricle (27) were made as described previously. Na,K-ATPase from the renal medulla purified by extraction with SDS was prepared as described previously (18).

**Proteolytic Digestion**—Proteolytic fingerprinting (Fig. 2) was done by the method of Cleveland et al. (28). Briefly, Na,K-ATPase α1 subunit was visualized in unfixed gels by immersion in 0.6 M KCl and cut out with a razor blade. Pieces of gel with the α1 subunit were loaded onto a gel, a gradient of 10-20% polyacrylamide, and protease solution in stacking gel buffer (with 0.1% SDS and glycerol) was layered over them. Proteolysis was allowed to proceed during gel electrophoresis; the extent of protolysis was controlled by varying the amount of protease used for a constant amount of α1 subunit, as specified in the figure legends. Staphylococcus aureus V8 protease (Endoprotease glu-C) was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Boehringer Mannheim, and papain was obtained from Calbiochem, Malvern, PA. Apparent molecular weights of the resulting fragments were calculated with the aid of molecular weight marker kits 7B and 17B obtained from Sigma.

Selective digestion of the undenatured, purified rat renal Na,K-ATPase with bovine trypsain-TPCK (Boehringer Mannheim) in the presence of K+ was performed essentially as described by Farley et al. (29). Na,K-ATPase was 1 mg/ml in 0.3 M sucrose, 15 mM KCl, 1 mM EDTA (Tris salt), 20 mM Tris-Cl, pH 7.2, and trypsain-TPCK was added to a final concentration of 7.3 μg/ml. Digestion was performed at 37 °C, and aliquots were taken at intervals and quenched with a 5-fold excess of soybean trypsin inhibitor (Sigma). Samples were also treated with phenylmethylsulfonyl fluoride at 1 μM/ml, before being dissolved in SDS for electrophoresis on gels of 7.5% polyacrylamide.

**Binding of Antibody to Enzyme Bound to Plastic**—Titer determinations were performed on Na,K-ATPase preparations, either 3 μg well of microsomes or 1 μg well of purified enzyme, adsorbed to Immulon 2 Removawell wells (Dynatech Laboratories, Inc., Alexandria, VA). After adsorption of the antigen, wells were quenched with Tris- or phosphate-buffered saline solutions containing 1% bovine serum albumin, and washed with saline containing 0.1% bovine serum albumin. Antibody-containing hybridoma culture supernatants were diluted as appropriate in saline solution containing 1% bovine serum albumin, incubated in the wells for 1 h, and washed. 125I-Labeled sheep-anti-mouse IgG (DuPont-New England Nuclear) diluted in saline line with 1% bovine serum albumin was then incubated in the wells for 1 h, washed, and counted with a γ counter.

**Results**

The Na,K-ATPase Is the Antigen Recognized by McK1—The monoclonal antibody McK1 was the result of fusing a mouse myeloma cell line with cells from the spleen of a mouse immunized with the purified, membrane-associated Na,K-ATPase (α1 and β) isolated from the rat renal medulla. The hybridoma-containing wells were screened first for binding to undenatured Na,K-ATPase and second for binding to its subunits, separated by electrophoresis in SDS and blotted onto nitrocellulose. The antibody is of the heavy chain subclass IgG1.

Fig. 1 demonstrates the apparent specificity of the antibody for the α1 isozyme of the catalytic subunit of the Na,K-ATPase. Three identical electrophoretic blots, which were stained with different antibodies, are shown. As marked with arrowheads on the left, each blot included prestained molecular weight markers. Each blot contained membrane samples from the newborn rat cardiac ventricle (V), axolemma from the adult brain stem (A), the renal medulla (K), and the cortex of the brain (B). The blot on the left was stained with a mixture of two previously described (18) rabbit antisera to the Na,K-ATPase, K2 and Ax2, to stain both the α1 and α(-) subunits, which are marked with double arrows. The β subunits were also stained and migrated with mobilities equivalent to molecular weights of 40,000–60,000 daltons. The blot in the middle was stained with McK1, and only the α1 subunit type was stained, not the α(-) of ventricle, axolemma, or brain. The blot on the right was stained with the sheep-anti-mouse IgG alkaline phosphatase conjugate that was used as the second antibody; it can be seen that this second antibody was responsible for the faint bands that were stained when McK1 was used.

Further evidence that McK1 was binding to the Na,K-
ATPase α1 subunit and not to a contaminant of the same electrophoretic mobility was obtained from its ability to bind to proteolytic fingerprints. As shown in Fig. 2, the α1 subunit from rat renal Na,K-ATPase was digested by S. aureus V8 protease or papain during gel electrophoresis by the method of Cleveland et al. (28). Nitrocellulose blots were prepared after electrophoresis in wide lanes of 10–20% polyacrylamide gradient gels. Strips were then cut from the blots and stained with antibodies, McK1, and three different rabbit polyclonal antibodies raised against rat kidney Na,K-ATPase: K1, K2, and K3.

McK1 clearly bound to fewer polypeptide fragments than any of the antisera, as expected for a monospecific reagent. McK1 stained a prominent subset of the fragments stained by the three antisera. The three polyclonal antisera stained different subsets of the smallest fragments obtained by digestion with the V8 protease, which suggests that they each have antibody populations specific for different stretches of sequence. This result is not surprising since the three antisera are known to have different levels of cross-reactivity with the cytochemistry (31) and the histochemical reaction of the K⁺-stimulated p-nitrophenylphosphatase (32).

**Fine Specificity Mapping of the McK1 Binding Site**—The use of fine specificity analysis to map the location of an antibody binding site is reasonable only if the antibody in question recognizes principally a stretch of sequential amino acids and not a more complex determinant dependent on tertiary structure. The fact that McK1 recognized the Na,K-ATPase α1 subunit after gel electrophoresis in SDS and electrophoretic blotting to nitrocellulose suggested that it should recognize such a linear sequence, but additional efforts were made to see if the antigenicity of the protein could be destroyed by even harsher denaturation conditions. Electrophoretic blots of gels were prepared on both nitrocellulose and polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Strips from the blots were subjected to 120 °C, 22-pound pressure for 30 min in empty glass Petri dishes in an autoclave, or alternatively, strips of the polyvinylidene difluoride blots were treated with 70% trifluoracetic acid for
FIG. 2. Proteolytic fingerprints of α1 stained by antibodies. Blots were prepared from 10–20% polyacrylamide gradient gels containing Na,K-ATPase rat α1 subunit digested by S. aureus V8 protease or papain during gel electrophoresis. The α1 subunit (100 μg) was cut from a prior gel and loaded onto wide lanes of the present gel, along with 33 ng of V8 protease or 22 pg of papain. After electrophoresis, the wide lanes were electroblotted onto wide pieces of nitrocellulose. Narrow strips were then cut, and each was stained with a different antibody: lanes 1, McK1; 2, K1; 3, K2; 4, K3. The position of the undigested α subunit is marked with arrows. A considerable amount of α aggregated and accumulated at the top of the gel.

The proteolytic breakdown fragments showed as lower molecular weight bands, whose average size decreased when the concentration of protease was increased in other experiments. Each antibody source showed related but not identical patterns of staining. The smallest fragment routinely detected by McK1 was about M, 20,000; the rabbit antisera stained fragments as small as M, 5,000, as determined by comparison with CNBr fragments of myoglobin.

5 min, and then washed with Tris-buffered saline. These treatments did not elute the Na,K-ATPase α1 subunit, as detected with Coomassie Blue stain, nor did they abolish subsequent McK1 binding in Tris-buffered saline (data not shown). We conclude that it is extremely unlikely that McK1 requires tertiary structure for its binding.

For fine specificity analysis, we prepared membrane fractions enriched in the Na,K-ATPase α1 subunit, as detected with Coomassie Blue stain, nor did they abolish subsequent McK1 binding in Tris-buffered saline (data not shown). We conclude that it is extremely unlikely that McK1 requires tertiary structure for its binding. For fine specificity analysis, we prepared membrane fractions enriched in the Na,K-ATPase α1 subunit, as detected with Coomassie Blue stain, nor did they abolish subsequent McK1 binding in Tris-buffered saline (data not shown). We conclude that it is extremely unlikely that McK1 requires tertiary structure for its binding. For fine specificity analysis, we prepared membrane fractions enriched in the Na,K-ATPase α1 subunit, as detected with Coomassie Blue stain, nor did they abolish subsequent McK1 binding in Tris-buffered saline (data not shown). We conclude that it is extremely unlikely that McK1 requires tertiary structure for its binding.
Fig. 4. McK1 recognition of Na,K-ATPase from different species. Each panel, A–F, represents a different blot or Coomasie-stained portion prepared from six otherwise identical polyacrylamide gels. The lanes contained 10 µg of protein of membranes from: 1, rat kidney; 2, mouse kidney; 3, chicken kidney; 4, Torpedo electric organ; 5, dog kidney; 6, monkey kidney (Macaca fascicularis); 7, sheep kidney; 8, pig kidney; 9 and 10, human kidney, 10 and 30 µg. Panels A–D were stained with McK1 hybridoma culture supernatant diluted as follows: A, 1:10; B, 1:100; C, 1:1000; D, 1:10,000. Panel E was stained with the alkaline phosphatase-conjugated goat-anti-mouse IgG secondary antibody alone. The arrow marks the position of migration of the α1 subunit. It can be seen that some stain in the mouse kidney samples and faint bands in the electric organ samples were due to the second antibody alone. Panel F was an identical polyacrylamide gel stained with Coomassie Blue.

A blot was incubated in the sheep-anti-mouse IgG second antibody alone; it is evident that some mouse-specific determinants were detected by the anti-mouse IgG second antibody, and one of them migrated just in front of the Na,K-ATPase α1 subunit. The affinity of McK1 for ostensibly undenatured Na,K-ATPase was tested by measuring its ability to bind to membrane preparations adsorbed to Immulon plastic wells. No binding to chicken, Torpedo, dog, sheep, or pig Na,K-ATPase was detected (data not shown). The binding to rat, mouse, monkey, and human kidney membrane preparations is shown in Fig. 5A. The binding to rat kidney membranes showed the highest titer and the highest total amount of binding; binding to the membranes from the other species was of lower titer as well as lower amount at the highest antibody concentration tested. The graphs were scaled to 100% to make it more obvious that the differences between species reflected differences in antibody affinity and not in the amount of antigen adsorbed to the plastic.

To assess the difference between the binding sites for McK1 on rat, mouse, monkey, and human Na,K-ATPase with completely undenatured enzyme, a competition assay was employed (Fig. 5B). Purified rat kidney α1 Na,K-ATPase was adsorbed to the wells as the binding antigen, while membrane preparations from rat, mouse, monkey, and human kidneys were used in the solution phase to compete for McK1. Different concentrations of the competing antigen were preincubated with a constant concentration of antibody for 1 h prior to exposing the mixture to the antigen adsorbed to the wells. As seen in Fig. 5B, rat kidney membranes competed effectively for binding to rat kidney Na,K-ATPase, but mouse, monkey, and human kidney membranes had little ability to compete at the concentrations used.

The following criteria were then used to search the primary amino acid sequences of the Na,K-ATPases for potential binding sites for McK1. The binding site must contain a short linear stretch of amino acids which is present in rat α1, homologous but slightly different in human α1, and different enough in rat αII, rat αIII, sheep α1, pig α1, chicken α1, and Torpedo αI plausibly to account for a lack of binding. Similar criteria have been used with success to predict antibody binding sites in other proteins (21). The aligned sequences are presented in Fig. 6. Complete sequences for mouse, monkey, and dog α1 are not presently available.

Examination of the aligned sequences revealed only two candidate sites for McK1 binding which are shown in Table I. All other sites in the sequence could be ruled out because of their identity with an amino acid sequence in one or more of the Na,K-ATPases that were not recognized by the anti-
FIG. 6. Comparison of Na,K-ATPase α-related sequences. Amino acid sequences, all deduced from cDNAs, were taken from the indicated references. The numbering is all-inclusive, i.e. based on the best alignment regardless of insertions and deletions, and thus may not correspond exactly to the numbering given in the original reports. Identical amino acids are represented by dashes, while gaps represent the absence of an amino acid.
body. The first site begins 20–21 amino acids from the N terminus (15–16 amino acids from the mature N terminus after proteolytic processing), while the second site begins at amino acid 496, just on the N-terminal side of the fluorescein isothiocyanate binding site. A potential third site carboxy to site 2, CSSILLSGK, is identical in rat and human and different in all other species. The McK1 binding site should not be identical in rat and human, however, since human Na,K-ATPase does not compete for binding to rat Na,K-ATPase with high affinity.

**Table I**

<table>
<thead>
<tr>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 2 Ref.</th>
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<tbody>
<tr>
<td>Rat αI</td>
<td>G D K K S K K A K K K N P N A S E P K H L L</td>
<td>10</td>
</tr>
<tr>
<td>Sheep αI</td>
<td>G D K K K A K K N A N A G E P R H L L</td>
<td>3</td>
</tr>
<tr>
<td>Pig αI</td>
<td>G D K K K A K K K N P N T A E P R H L L</td>
<td>5</td>
</tr>
<tr>
<td>Chicken αI</td>
<td>G T K K K K A K N A P E S E R Y S L</td>
<td>20</td>
</tr>
<tr>
<td>Torpedo αI</td>
<td>N A K N S K R S K S H E N D K A D S R Y L</td>
<td>4</td>
</tr>
<tr>
<td>Rat αII (+)</td>
<td>E N G G G K E Q K H E R E D S P Q S H V L</td>
<td>10</td>
</tr>
<tr>
<td>Rat αIII</td>
<td>S P K K S E K K A E T E D P N D N R Y L</td>
<td>10</td>
</tr>
</tbody>
</table>

The **underlined amino acids** are those that meet the criteria for species and isozyme specificity for McK1 in the rat and human α1. Further experiments demonstrated that site 1 is the site actually recognized by the antibody. In sheep and pig, two of its critical amino acids are deleted; in chicken α1, Torpedo α1, and rat αIII, one amino acid is deleted. In Torpedo α1, there is a conservative substitution. In chicken α1, Torpedo α1, and rat αIII there are substitutions of the aspartate residue as well. In rat αIII there is little homology at all at this site. The numbering of the amino acids in the homologous sites does not correspond when different sequences are compared because of insertions and deletions. For reference, however, site 1 in rat α1 begins at amino acid 20 (starting from the initiator methionine), while site 2 begins at amino acid 496.

**Location of the Epitope on the Linear Peptide Map—Digestion** of the native Na,K-ATPase by trypsin is known to occur at several restricted and well defined sites (33, 34), and the fragments that are generated have been aligned and form the basis of a linear polypeptide map (1, 33). Fig. 7A summarizes the linear map and shows the location of identified sites of proteolysis and covalent modification. We took advantage of this selective digestion to determine which of the two candidate sites of Table I is the binding site for McK1. In the presence of K+, trypsin preferentially attacks an arginine at amino acid 448, called trypsic site T1 (1). This divides the protein into an N-terminal fragment of M, 48,312 and a C-terminal fragment of M, 64,261 (34). The major tryptic sites are conserved between species, and so all α1 types are expected to give similar digestion patterns. Upon gel electrophoresis in SDS, these fragments typically migrate with apparent molecular weights of 41,000–48,000 and 53,000–62,000, respectively; the faster-than-predicted migration (like that of the intact α subunit) is thought to be due to excess SDS binding to hydrophobic regions. Site 1 for McK1 should be found in the smaller fragment, and site 2 in the larger one, as indicated in Fig. 7A.

The results of tryptic digestion of the native rat α1 enzyme in the presence of K+ are shown in Fig. 7B. In the experiment shown, trypsin was incubated with the Na,K-ATPase at 37 °C, and aliquots were removed for quenching with soybean trypsin inhibitor at 3, 6, 9, and 12 min. Samples of digested enzyme were electrophoresed in SDS on a polyacrylamide gel, and two identical electrophoretic blots were prepared. The blot on the left in Fig. 7B also contained the Sigma 7B prestained molecular weight markers. The blot on the left was stained with K1, a rabbit antiserum raised against the rat kidney α1 subunit. This antiserum stained the undigested α1 subunit, a doublet at 52,500 and 49,600 which we take to be the C-terminal fragment, a band at 42,000 which we take to be the N-terminal fragment, and several products of secondary digestion. All of the same fragments were seen on identical gels stained with Coomassie Blue (data not shown) and, conversely, all of the fragments visible with Coomassie Blue were stained by K1. Similar results were obtained in experiments in which the concentration of protease was varied instead of the length of digestion. The cleavage at the trypsic site T1 is known to be very rapid (1), and appears to be nearly complete already after 3 min of digestion.

**Fig. 7.** Mapping by tryptic digestion of undenatured Na,K-ATPase. A, linear map of the α1 subunit of the Na,K-ATPase. The linear map and the position of the major tryptic fragments is taken from the literature (1, 33, 34). T1(K) is the site digested by trypsin most readily in the presence of K+. T2 is digested both in Na+ and in K+, but more rapidly in Na+. T3(Na) is a second site digested more slowly in Na+. C is a site digested by chymotrypsin. P* is the aspartate residue that is covalently phosphorylated from [γ-32P]ATP in the active site (33). Fluorescein isothiocyanate (FITC) (35), fluoresulfonyl benzoyl adenosine (FSBA) (36), and chloroalkyl benzylamine ATP (C1ATP) (37) are covalently bound reagents thought to label areas near the ATP binding site. Benzimidazolyl phenylmaleimide (BIPM) is a fluorescent probe used for studies of conformation changes (38). The N- and C-terminal fragments generated by cleavage at the T1(K) site are marked 42 K and 53 K, respectively. Sites 1 and 2 from Table I are marked on the fragments. B, undenatured Na,K-ATPase was digested with trypsin-TPKC at a ratio of 0.02 µg trypsin/µg Na,K-ATPase in the presence of K+ at 37 °C. Samples were removed at 3, 6, 9, and 12 min, and the proteolysis was stopped with excess soybean trypsin inhibitor. The samples were divided between three identical polyacrylamide gels and electrophoresed, and two gels were blotted onto nitrocellulose while the third was stained with Coomassie Blue (not shown). The blots were stained with either rabbit antiserum K1 or with McK1, as indicated. K1 stained sites in both the N- and C-terminal fragments of the Na,K-ATPase, and so it was used to identify the proteolytic products. The lane at the far left shows prestained molecular weight markers of M, 116,000, 84,000, 58,000, 48,500, 36,500, and 26,500 (Sigma 7B). The position of undigested α1 subunit is marked by α. T1 marks the higher and lower molecular weight products of cleavage at the T1 tryptic site. T2a, b, c, d, and e mark products of secondary digestion. In the panel on the right, McK1 stained only the undigested α1 subunit and the M, 42,000 N-terminal fragment.
Secondary digestion proceeded more slowly. In the presence of K⁺, the Na,K-ATPase is known to be cleaved more slowly at a site or sites close to the N terminus called T2 (1, 33). Previous determinations of the N-terminal amino acids of the fragments resulting from trypptic digestion have identified at least two alternative T2 sites (33, 34); as a lysine-rich region, the N terminus potentially could be cleaved at several sites. The digestion products observed here were tentatively assigned the names T2a through T2e in Fig. 7B. Five likely sites of secondary cleavage can be predicted whose true molecular weights should match the experimentally determined molecular weights very well (amino acids 25, 38, 46, 55, and 68 of Fig. 6). These sites include the two sites (T2a and T2b) previously determined by N-terminal analysis (33, 34), but they have not been experimentally verified in the present case. The data are consistent with the premise that secondary tryptic digestion of the N-terminal fragment of the rat a1 Na,K-ATPase can occur at five nearly equivalent sites. Digestion at these five sites did not appear to be sequential, since the pattern stabilized after 9 min rather than continuing toward smaller and smaller fragments. Secondary digestion occurring at a site near the C-terminal of the M₄, 42,000 N-terminal fragment may occur, but the identity of the rat, human, and pig sequences in this region rules it out as a binding site for McK1.

When an identical blot was stained with McK1, only the undigested a1 subunit and the M₄, 42,000 N-terminal fragment were labeled (right panel of Fig. 7B). Neither the C-terminal fragment doublet nor the products of secondary digestion were labeled. This strongly implicates site 1, Asp-Lys-Lys, as the site recognized by the antibody.

It is notable that the K₁ rabbit antiseraum stained a protein of apparent M₄, 43,000 that first appeared at 6 min and whose concentration on the blot increased with time of digestion. This band virtually comigrated with the intact M₄, 42,000 N-terminal fragment, but was not stained by McK1. In the photograph, the band appears more gray than black; in the original blot stained for alkaline phosphatase, it appeared more pink than blue, and strongly resembled the pinkish stain of the C-terminal fragment doublet. The pink hue probably reflects a lower density of alkaline phosphatase reaction product. This band is likely to be a product of secondary digestion of the C-terminal fragment. Its presence might explain why Farley et al. (29) saw apparent binding of a monoclonal antibody to both N- and C-terminal fragments of the Na,K-ATPase; the binding may have actually been to the C-terminal fragment and its secondary digestion product. The lower M₄ fragments (labeled T2a-T2e in Fig. 7B) have been interpreted in the present paper as being derived from the N-terminal fragment, primarily on the basis of their blue, rather than pink, stain by K₁. The available experiments do not rigorously rule out the possibility that some of them may be derived instead from the C-terminal fragment, however.

Binding of the Antibody to Oriented Resealed Kidney Tubule Vesicles—To determine the transmembrane orientation of the McK1 binding site, we prepared resealed right-side-out vesicles from the rat renal medulla on gradients of metrizamide (39, 40). Such vesicles have been shown to be oriented largely with the ATP hydrolyzing site of the Na,K-ATPase on the interior, where it does not have access to ATP in the assay solution. Mild treatment of our vesicles with detergent (SDS) resulted in a 12–16-fold increase in Na,K-ATPase activity, indicating that the vesicles were 92–94% right-side-out and sealed to small molecules. When sealed right-side-out vesicles were adsorbed directly to Immulon plastic wells and assayed for their ability to bind McK1, we obtained the unexpected result that substantial binding was obtained even in the absence of detergent pretreatment. Either pretreatment of the vesicles with SDS prior to adsorption to the wells or post-treatment after adsorption to the wells gave the same result: a 1.4–1.7-fold increase in McK1 binding, not the expected 12–16-fold increase. At first glance this suggested that McK1 might bind to a determinant exposed on the outside of the sealed right-side-out vesicles. As a control, however, we investigated the ability of a rabbit antiserum against a known intracellular determinant to bind to sealed right-side-out vesicles under the same conditions. The antiserum was against the synthetic peptide KGAPER (41), and was the generous gift of K. Xu and J. Kyte of the University of California, San Diego. This antibody bound equally well to the adsorbed vesicles whether or not they were pretreated with detergent. Our conclusion was that the vesicles had become unsealed and intravesicular determinants had become exposed when the vesicles interacted with the hydrophobic surface of the Immulon plastic wells. The vesicles had been diluted from the metrizamide density gradient medium into isotonic solutions prior to adsorption to the wells; however, dilution into buffered saline, buffered sucrose solution, or ATPase activity assay medium alone without SDS treatment did not result in a stimulation of ATPase activity, indicating that the experimental conditions were unlikely to have lysed the vesicles before they were adsorbed to the plastic wells.

To obtain a more accurate assessment of the ability of McK1 to bind to sealed right-side-out vesicles, we then performed a solution-phase competition assay. Antibody was mixed with vesicles and allowed to bind to them in solution, and the residual unbound antibody was then measured by its ability to bind to purified Na,K-ATPase adsorbed to plastic wells. The experiment was done with sealed vesicles and with vesicles opened by prior treatment with low concentrations of apparent M₄, 43,000 that first appeared at 6 min and whose concentration on the blot increased with time of digestion. This band is likely to be a product of secondary digestion of the C-terminal fragment. Its presence might explain why Farley et al. (29) saw apparent binding of a monoclonal antibody to both N- and C-terminal fragments of the Na,K-ATPase; the binding may have actually been to the C-terminal fragment and its secondary digestion product. The lower M₄ fragments (labeled T2a-T2e in Fig. 7B) have been interpreted in the present paper as being derived from the N-terminal fragment, primarily on the basis of their blue, rather than pink, stain by K₁. The available experiments do not rigorously rule out the possibility that some of them may be derived instead from the C-terminal fragment, however.

**FIG. 8.** Competition assay for McK1 binding to right-side-out kidney vesicles. McK1 (diluted 1:600) was preincubated with different concentrations of sealed right-side-out kidney vesicles (Ⅰ) or vesicles pretreated with SDS to open them (Ⅱ), prior to incubation in wells containing adsorbed purified Na,K-ATPase from the rat kidney. The SDS pretreatment was done as follows. Right-side-out vesicles containing 36 μg of protein in 60 μl were preincubated with 3 mM ATP, and 1 μl containing 10 μg of SDS was added. After 15 min at room temperature, the sample was diluted with 50 μl of water and centrifuged for 10 min in an Airfuge (Beckman). The pellet was resuspended in 60 μl of buffer. Although some non-Na₄,K-ATPase protein may have been removed during the detergent treatment, we assumed that purification was negligible, that recovery of the Na,K-ATPase was close to 100%, and that the final protein concentration was unchanged by the detergent pretreatment. Opened vesicles competed for McK1 at 15–20-fold lower concentrations than did sealed vesicles. The arrows indicate the concentrations at which half-maximal competition occurred.
of SDS; after the SDS pretreatment, the vesicles were centrifuged to separate them from excess SDS and resuspended in the original volume of detergent-free buffer. The ATPase activity of the detergent-pretreated vesicles was stimulated 16-fold. The result of the competition experiment is shown in Fig. 8. The detergent-pretreated vesicles competed with the adsorbed Na,K-ATPase for McK1 binding half-maximally at 1.3 μg of protein, while the sealed vesicles competed half-maximally at 23 μg of protein. This is equivalent to a 17-fold increase in ability to compete after detergent pretreatment, very similar to the stimulation of ATPase activity. To control for the effects of residual SDS in the detergent-stimulated vesicles, we also performed the experiment in the presence of amounts of SDS equivalent to those used to open the vesicles, much more SDS than actually present in the experiment. We found that even at the highest possible concentration of residual SDS, there was no more than a 10% reduction in McK1 binding in the controls (data not shown). The results of Fig. 8 are most easily interpreted by the conclusion that the McK1 binding site is exposed on the cytoplasmic surface of the cell.

**DISCUSSION**

**Antibody Specificity and N-terminal Topography—** McK1 is a monoclonal antibody against the Na,K-ATPase that is specific for the α1 isozyme. It bound to native or denatured enzyme, and recognized some, but not all, of the animal species tested. Fine specificity mapping revealed only two possible sites where amino acid insertions and substitutions could account for its specificity, and peptide mapping located the antibody binding site in the N-terminal half of the protein. The fact that McK1 binding was abolished after the first step of secondary tryptic digestion strongly supported the conclusion that the antibody binding site lies within the first 2000 daltons of the N terminus. The deduced site includes Asp-Lys-Lys-Ser-Lys-Lys-Ala-Lys-Lys, which could plausibly account for its cross-reactivity.

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The a1 subunits of other species (sheep and pig) contain the homologous sequence Asp-Lys-Lys-Gly-Lys-Lys-Lys-Ala-Lys-Lys, which could plausibly account for its cross-reactivity. The α1 subunits of other species (sheep and pig) contain the similarly homologous sequence (Lys)-Lys-Lys-Ala-Lys-Lys, and yet they are not recognized by McK1. For this reason, we have inferred that the aspartate residue may form an important component of the antibody binding site, and that its substitution with a lysine residue is not permitted. Examination of the sequences in Table 1 shows that the other α subunit types that are not recognized by the antibody have even more significant differences.

Farley et al. (29) have presented evidence that several polyclonal and monoclonal antibodies have binding sites clustered in the N-terminal half of the Na,K-ATPase, based on their ability to bind to the major tryptic fragments. Kyte et al. (41) have made an antibody against the highly conserved Na,K-ATPase sequence KGAPER. Ball and his colleagues (42, 43) have determined the approximate site of binding of several monoclonal antibodies by their ability to recognize synthetic peptides corresponding to a few selected regions of the α1 subunit, and they have additionally prepared polyclonal antisera against some of the synthetic peptides. While the antibodies against peptides are particularly valuable, in most cases it still cannot be explicitly predicted which exact amino acids comprise their binding sites. Fine specificity mapping should be widely applicable for the analysis of Na,K-ATPase antigenic sites.

It has been generally assumed that the N terminus of the Na,K-ATPase is exposed at the intracellular surface, although the evidence is actually indirect and in some instances conflicting. In opened membrane fragments, trypsin (in the presence of Na+) causes a biphasic inactivation of activity with rate constants that appear to correlate with rapid and slow cleavage at two different sites, T2 near the N terminus and T3 at about residue 262-263 (34, 44). The sites and the molecular weights of the corresponding fragments on SDS gels have been determined only in opened membrane preparations, not permitting assignment to the cytoplasmic or extracellular faces. Evidence that the sites are cytoplasmic came from the proteolysis of enzyme reconstituted into lipid vesicles (45). Only pumps that are oriented inside-out in sealed vesicles participate in Na+ transport activity. With trypsinization, a biphasic loss of Na+ transport activity was seen that was identical to the loss of Na,K-ATPase activity seen in opened membrane preparations, and it was inferred that the proteolysis was occurring at sites T2 and T3. The actual gel electrophoretic mobility of the proteolyzed enzyme fragments was not examined in the experiment with reconstituted vesicles, however (45).

A puzzling observation was published by Forbush (39) in his description of the properties of sealed right-side-out kidney vesicles. He observed that Na,K-ATPase activity was lost when right-side-out vesicles were treated with bovine trypsin (from four different commercial sources), but not when treated with porcine trypsin (two different sources), which indicates the presence of an extracellularly oriented proteolytic site. All tryptic digestion of the Na,K-ATPase reported in the literature to date has been with bovine trypsin.

In intact human red blood cells, the Na,K-ATPase is insensitive to inactivation by trypsin, suggesting that there are no tryptic sites on the extracellular surface or that they are masked by other membrane components (46). Although there is not enough Na,K-ATPase in human red blood cells to permit direct visualization of the Na,K-ATPase proteolytic fragments, Chin and Forgac (47) showed that it is possible to visualize the phosphorylated N-terminal fragment by autoradiography after proteolytic digestion of inside-out red blood cell vesicles. The 41,000-dalton N-terminal fragment was seen in trypsinized inside-out vesicles, but the authors stated that they did not see the expected secondary decrease in apparent molecular weight of this fragment (presumably at the T2 site) with higher trypsin concentrations. It is possible that digestion at the T2 site occurred more rapidly than at the T1 site under their conditions, and in fact they reported seeing a small shift in the mobility of the otherwise undigested α subunit. It was also possible, however, that the failure to see secondary digestion of the N-terminal fragment was due to its being sequestered at the extracellular (intravesicular) surface. The uncertainties in the above data, and the absence of definitive correlation of activity loss with proteolysis at the T2 site in the work of Karlish and Pick (45), left it possible that the N-terminal T2 tryptic site was actually exposed on the outside of the cell and that, when cleaved with bovine trypsin, the Na,K-ATPase was inactivated.

The present results provide more direct evidence that the N terminus of the Na,K-ATPase is exposed at the cytoplasmic surface. Chin and Forgac (47) and Karlish and Pick (45) were correct in their interpretation of their data. The curious observation of Forbush (39) is still unexplained, but it may involve a different proteolytic site at some other extracellular location.

**Prediction of Secondary Structure at the N Terminus**—The fact that McK1 binds to undenatured enzyme indicates that its binding site is exposed on the surface of the protein. This result is consistent with the observation of Ball and Loftice
that antibodies raised against a synthetic peptide including the same region of the sheep sequence (Lys-Lys-Lys-Ala-Lys-Lys-Glu-Agr-Met-Met-Glu-Leu-Lys-Lys) also bind to both native and denatured Na,K-ATPase. The N terminus of the Na,K-ATPase isozymes shows a marked divergence in amino acid sequence (10). In fact the N terminus is one of only several sites where nonconservative substitutions predominate in the Na,K-ATPase sequences (see the sequence comparison in Fig. 6). The first 20–30 amino acids, including the McK1 binding site on α1, can be considered N-terminal extensions of the Na,K-ATPase on the basis of a predictive analysis of structure.

The Chou and Fasman analysis (48) was used to analyze and compare possible secondary structure configurations for the N termini of rat α1, αII, and αIII (data not shown). Such predictions are based on frequencies of structures adopted in proteins whose crystalline structures have been determined, and are only accurate about two-thirds of the time because they do not take into account long-range interactions and other forces inherent to tertiary structure. The predictions do, however, serve as an interesting framework for considering the possible structure and role of the N termini. No β sheet structure is predicted for the first 50 amino acids of the Na,K-ATPase. A region of very high homology begins at amino acid 30 in αI, amino acid 28 in αII, and amino acid 20 in αIII. Starting at these points, there are only very conservative substitutions for 20 or more amino acids, and all three iso- 
ymes are predicted to have α-helical structure. Closer to the N terminus, however, where there is much more divergence, the probability estimates give different patterns of secondary structure for the three different sequences. The αI isozyme is calculated to be most likely to adopt an α-helix starting at residue 9, and continuing right through the McK1 binding site and into the conserved region. The αII isozyme is predicted to have α-helix starting at residue 5, but broken by two β turns; the second β turn is predicted to be at or just before the sequence that replaces the McK1 binding site. The αIII isozyme has a much shorter N-terminal sequence, with no secondary structure prediction until amino acid 10, where a single β turn is predicted, again close to the sequence that replaces the McK1 binding site.

The predictions are based on probability estimates whose thresholds are set by the investigator. It is particularly implausible to have an α-helix extending for over 40 amino acids in αI, and several positions of relatively low α-helical potential are likely to be breaks in reality. What may be of most significance for the structure of the Na,K-ATPase is simply that the divergent sequences do not fit easily into a homolo-
gous folding pattern.

The lack of conserved secondary structure would be consistent with the premise that surface-exposed stretches of amino acids are not as constrained in the arrangement of their hydrogen bonds as buried stretches. Although it may be shown eventually that the three isozymes achieved homolo-
gous folding with very different amino acid sequences in this region, it is more parsimonious to predict that the N termini form solvent-exposed segments with different structures. The possible roles of these segments is not known, but proteolysis at least one of the possible T2 sites results in the partial loss of Na,K-ATPase activity (44, 45), and the data of Jorgensen and Collins (34) suggest that the N terminus of the αII isozyme has an influence on Na⁺ affinity or Na⁺-induced conformation change, since proteolysis at the T2b site has an effect on Na⁺-induced changes in the fluorescence of fluores-
ccein isothiocyanate. Such observations argue that the N-terminal segment is not present merely to provide a docking site for regulatory or cytoskeletal proteins, but that it may play a more direct role in the function of the enzyme, in ways that can be expected to differ between the isozymes. McK1 does not inhibit the hydrolysis of ATP, but more subtle effects on Na,K-ATPase function have yet to be tested.

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