Topological Disposition of Lysine 943 in Native Na^+/K^+-Transporting ATPase†

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ABSTRACT: Because of the conflicting conclusions that have been reached regarding the location of the two putative membrane-spanning segments from cysteine 911 through isoleucine 929 and from isoleucine 946 through cysteine 964 in the α subunit of native ovine Na^+/K^+-transporting ATPase, the disposition of lysine 943 with respect to the plane of the lipid bilayer was investigated. Sealed, right-side-out vesicles were modified with pyridoxal phosphate and $Na[^3H]BH_4$ in the presence and absence of saponin, a reagent that creates holes in the membranes. Modified α polypeptide was isolated, and digested with trypsin and chymotrypsin to release the desired peptides, QQGMK and QQGMK([^{3}H]pyr)NK (where [^{3}H]pyr designates the modification on lysine 943). These peptides, after cyclization of their amino-terminal glutamines, were isolated with an immunoadsorbent specific for the amino-terminal sequence pyroglutamyl-QGM- followed by high-pressure liquid chromatography on a C-18 reverse phase column. Comparisons were made of the extent of incorporation of radioactivity into lysine 943 between sealed vesicles and sealed vesicles pretreated with saponin. An increase in incorporation into lysine 943 of 5-fold to 18-fold was seen in vesicles pretreated with saponin prior to the modification with pyridoxal phosphate. This increase in incorporation is consistent with a cytoplasmic location for lysine 943. This conclusion places the residues on the carboxy-terminal side of the putative membrane-spanning segment from cysteine 911 through isoleucine 929 and the amino-terminal side of the putative membrane-spanning segment from isoleucine 946 through cysteine 964 in the ovine α subunit on the cytoplasmic side of the membrane.

Na^+/K^+-Transporting ATPase is an integral, membrane-spanning protein that is responsible for the primary active transport of Na^+ and K^+ ions across the plasma membranes of all animal cells. In order for the ions to traverse the membrane, a pathway must exist through the protein. The most logical candidates to form this pathway are the membrane-spanning segments of the protein. In theory, the identification of such membrane-spanning segments should have computational and experimental solutions.

Many computational methods have been proposed to identify membrane-spanning segments based upon the average hydrophobicity of the residues in the segment (Argos et al., 1982; Kyte & Doolittle, 1982; Guy, 1984; Kuhn & Leigh, 1985; Engelman et al., 1986; Rees et al., 1989). Depending upon which of these computer algorithms is applied to Na^+/K^+-transporting ATPase, 6−10 segments are predicted to span the membrane. It should be noted that profiles of hydropathy can be misleading. Even though a segment has a high degree of hydrophobicity, it does not necessarily follow that this segment must span the membrane. For example, lactate dehydrogenase, a water-soluble protein, contains 1 of the most hydrophobic 19 amino acid segments known. The inability to distinguish a highly hydrophobic segment buried in the middle of a portion of the native structure of a protein outside the membrane from a segment spanning the membrane remains a problem.

One alternative approach to determining which segments of a protein span the membrane is to label them directly with a hydrophobic, uncharge reagent that partitions preferentially into the lipid bilayer and modifies hydrophobic amino acids within the hydrocarbon and then to isolate the peptides into which the label has been incorporated (Brunner & Semenza, 1981; Bayley & Knowles, 1980). Previous studies in our laboratory used the hydrophobic label [1-^{3}H]spiro(adamantane-4,3′-diazirine) to label Na^+/K^+-transporting ATPase covalently (Nicholas, 1984). Five labeled peptides were isolated from a tryptic digest of the modified enzyme. Each of these peptides contained 1 or 2 hydrophobic segments 20 amino acids in length and were assumed to be membrane-spanning segments. These experimental results, however, differed significantly from the computational predictions (Kyte, 1987), which differed among themselves. It follows that the identity of the actual membrane-spanning segments in Na^+/K^+-transporting ATPase remains uncertain.

Another approach to determine if a segment actually spans the membrane is to determine where the hydrophilic segments on either side of the putative membrane-spanning segment lie with respect to the plane of the bilayer. If residues on the amino-terminal and carboxy-terminal sides of a hydrophobic segment can be shown to reside on opposite sides of the membrane, the intervening segment must span the membrane. In this study, a combination of vectorial labeling and site-directed immunochemistry was used to determine the location of lysine 943 in the α subunit of ovine Na^+/K^+-transporting ATPase. Lysine 943 is a residue that lies in the hydrophilic region between two proposed membrane-spanning segments, cysteine 911 through isoleucine 929 and isoleucine 946 through cysteine 964. Its location defines the location of the carboxy-terminal side of the first segment and the amino-terminal side of the second.

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EXPERIMENTAL PROCEDURES

Materials. Adenosine-5′-triphosphatase, Amberlite XAD-4, 3,5-bis(acetamido)-2,4,6-triiodobenzoate, bovine serum albumin (BSA),[1] carboxypeptidase B, leucine aminopeptidase, phosphoramidone, pyroglutaminase, sodium borohydride, and trypsin that had been treated with *N*-(*p*-toluenesulfonyl)-L-phenylalanyl chloromethyl ketone were purchased from Sigma Chemical Co. Pyridoxal phosphate was purchased from either Sigma Chemical Co. or ICN Biochemicals. Amino acids protected at their α-amino positions with 9-fluorenylmethyloxycarbonyl (FMOC)[1] groups were purchased from either Beckman Corp., Bachem Corp., or Novabiochem Corp. Acetonitrile (high-pressure liquid chromatographic grade), dimethyl sulfoxide (DMSO),[1] and tris(hydroxymethyl)aminomethane (Tris)[1] were purchased from Fisher. Dicyclohexylcarbodiimide, 1,3-diisopropylcarbodiimide, 1-hydroxybenzotriazole hydrate, and triethanolamine were purchased from Aldrich Chemical Co. Affigel 10, Bio-Gel A-5M and A1.5M, protein A−agarose, and Dowex AG 1X2 resin were purchased from Bio-Rad Corp. Ecolume scintillation cocktail and ultrapure urea were purchased from ICN Biochemicals. Carboxypeptidase Y, Pronase, saponin, and thermolysin were purchased from Calbiochem Corp. Dimethyl pimelimidate was purchased from Pierce Chemical Co., Fruend's complete and incomplete adjuvants were purchased from Difco Laboratories, *p*-alkoxybenzyl alcohol resin (Wang's resin) was purchased from Bachem, and chymotrypsin was purchased from Worthington Biochemical Corp. Fresh ovine kidneys were purchased on the day of slaughter from Superior Meat Packing Co., Dixon, CA. Sodium [^{3}H]borohydride (Na[^{3}H]-BH_4) was purchased as a dry solid from New England Nuclear Corp. Imidazole was purchased from Sigma Chemical Co. and recrystallized from benzene and then from acetone. Sodium dodecyl sulfate (SDS) was purchased from Sigma Chemical Co. or Calbiochem Corp. and recrystallized from 95% ethanol (Burgess, 1969), and cholate was purchased from Sigma Chemical Co. and prepared by boiling with Norit A in 95% ethanol, filtering, and recrystallizing from 70% ethanol.

Peptide Synthesis. The peptides pyroglutamyl-QGMK (pyro-EQGMK),[1] pyro-EVFQQGMK, and pyro-EQGMKNKILIF were synthesized by solid-phase FMOC methods (Stewart & Young, 1984) using *p*-alkoxybenzyl alcohol resin (Wang's resin). The first amino acid, either N^{α}-FMOC-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine or N^{α}-FMOC-L-phenylalanine, was coupled to the resin with dicyclohexylcarbodiimide. The following protected L-amino acids were used to complete each synthesis: N^{α}-FMOC-L-isoleucine, N^{α}-FMOC-L-leucine, N^{α}-FMOC-N^{ϵ}-(*tert*-butyloxycarbonyl)-L-lysine, N^{α}-FMOC-L-asparagine, N^{α}-FMOC-L-methionine, N^{α}-FMOC-L-glycine, N^{α}-FMOC-L-glutamic acid, and L-pyroglutamic acid. The peptides were elongated at each step by the addition of the appropriate FMOC amino acid (6 mmol), diisopropylcarbodiimide (6 mmol), and hydroxybenzotriazole (6 mmol). The peptides were cleaved from the resin, and blocking groups were removed by treatment with 95% trifluoroacetic acid, 3% ethanedithiol, and 2% thioanisole for at least 4 h at room temperature. High-pressure liquid chromatography (HPLC)[1] on a C-18 reverse phase column was used to purify the peptides.

Modification of the Peptide Pyro-EQGMKNKILIF with Pyridoxal Phosphate. Peptide (10 mg) that had been purified by HPLC was resuspended in 100 μL of 0.1 M sodium phosphate, pH 8.0, and DMSO (100 μL) was added to help dissolve the peptide. Solid pyridoxal phosphate was added, and the pH was adjusted to 8.0 with 1 M NaOH. The final concentration of pyridoxal phosphate was approximately 0.3 M. After 30 min, a 2-fold molar excess of sodium borohydride was added. The mixture was stirred for 20 min at room temperature, and the reaction was stopped by dropping the pH to 4.0. The modified peptide was then isolated by HPLC on a C-18 reverse phase column.

Preparation of an Immunoadsorbent Specific to the Amino-Terminal Pyro-EQGM-. Polyclonal immunoglobulins were produced by coupling the synthetic peptide pyro-EQGMK to BSA (Walter et al., 1980) and immunizing rabbits with the resulting conjugate. Synthetic pyro-EQGMK (4 mg) was added to BSA (10 mg dissolved in 2.0 mL of 150 mM sodium chloride, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4). An equal volume of 1.0% glutaraldehyde (2.0 mL) was added, and the solution was stirred at room temperature for 1 h. The reaction was stopped by the addition of 40 mg of $NaBH_4$. After stirring overnight at 4 °C, the mixture was dialyzed against PBS, and the conjugate was submitted to amino acid analysis. It could be calculated that 9.0 nmol of peptide was bound to every nanomole of BSA. This conjugate was injected into White New Zealand rabbits. A 1:1 mixture of conjugate and Freund's complete adjuvant was prepared and injected subcutaneously and intramuscularly for the initial injection. Subsequent boosts were made with a suspension of conjugate in Freund's incomplete adjuvant.

An affinity column was prepared to isolate the desired immunoglobulins. The synthetic peptide pyro-EQGMK (19 mg) was dissolved in 0.1 M HEPES, pH 7.5, and added to 2 mL of succinylated agarose activated with *N*-hydroxysuccinimide (Affi-gel 10). The slurry was shaken overnight at 4 °C. The slurry was washed with buffer, suspended in 300 mM monoethanolamine for 1 h, and then washed once again with buffer, and a sample was taken for amino acid analysis. It was found that 2.6 μmol of peptide was coupled to each milliliter of resin.

Antiserum was passed over this affinity column to isolate polyclonal immunoglobulins specific to the sequence pyro-EQGM- as described in Dwyer (1991). The isolated immunoglobulins (1.5 mg in 3.5 mL) were added to protein A−agarose (1.5 mL), and the slurry was shaken overnight at 4 °C. The agarose was then washed with 0.2 M triethanolamine, pH 8.2, resuspended in 20 mM dimethyl pimelimidate, 0.2 M triethanolamine, pH 8.2, and stirred for 1 h at room temperature. It was then washed with 20 mM ethanolamine for 20 min followed by washing with 1 M acetic acid to remove unbound immunoglobulins. The capacity of the immunoadsorbent for the synthetic peptide pyro-EQGMK was determined to be 13 nmol.

[1] Abbreviations: SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; C-18, octadecylsilyl silca gel; FMOC, 9-fluorenylmethyloxycarbonyl; BSA, bovine serum albumin; PBS, phosphate-buffered saline (150 mM sodium chloride, 0.1 mM EDTA, 20 mM sodium phosphate, pH 7.4); EDTA, ethylenediaminetetraacetic acid; HEPES, sodium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; Na[^{3}H]BH_4, sodium [^{3}H]-borohydride; K(Hpyr), lysine modified by pyridoxal phosphate and sodium borohydride; pyro-E, pyroglutamyl; DTT, dithiothreitol; DMSO, dimethyl sulfoxide.

Preparation of Sealed, Right-Side-Out Vesicles and Open Fragments of Membranes Rich in Na^+/K^+-Transporting ATPase. Membranes rich in Na^+/K^+-transporting ATPase (microsomes) are isolated from ovine renal medullas by the method of Jørgensen and Skou, (1969) as modified by Kyte (1971) and described in Thibault (1993). The enzymatic activities of the microsomes were between 15 and 40 μmol of $HOPO_3^{2-}$ h^{-1} mg^{-1}. Right-side-out, sealed vesicles are prepared from these microsomes as described in Thibault (1993). The enzymatic activities of the sealed vesicles were between 20 and 40 μmol of $HOPO_3^{2-}$ h^{-1} mg^{-1}.

Latency Assay. Sealed vesicles are incubated with or without 0.4% saponin for 12 min at room temperature, and portions of each sample are removed and assayed for strophanthidin-sensitive Na^+/K^+-transporting ATPase activity (Kyte, 1971). A comparison of the enzymatic activity in saponin-treated and untreated vesicles reveals how tightly the vesicles are sealed.

Labeling of Sealed, Right-Side-Out Vesicles with Pyridoxal Phosphate. The labeling method of Ohkawa and Webster (1981) as described by Dwyer (1991) and Thibault (1993) was followed without modification.

Isolation of the α Polypeptide of Na^+/K^+-Transporting ATPase. The α polypeptide was isolated from either opened membranes or sealed vesicles by gel filtration in 0.1% SDS, and the dodecyl sulfate and urea were removed as described in Thibault (1993).

Proteolytic Digestion. Tryptic and chymotryptic digestions were performed in 0.1 mM calcium chloride, 0.1 M ammonium bicarbonate, pH 8.0. Trypsin was added at a ratio of 1:10 with respect to the amount of protein, the digests were incubated at 37 °C for 1 h, an additional amount of trypsin was added, and the digests were incubated for an additional hour. Chymotryptic digestions were carried out either at 0.05 mg mL^{-1} chymotrypsin or at a ratio of 1:10 with respect to substrate. Digestions were incubated for 1 h at 37 °C, an equal amount of chymotrypsin was added, and the digestion was continued for an additional hour. To inhibit these two serine proteinases, at least a 4-fold excess [4 g (g of protease)$^{-1}$] of soybean trypsin inhibitor was added. Thermolytic digestions were carried out in 1.0 mM calcium chloride, 0.1 M ammonium bicarbonate, pH 8.0. Thermolysin was added (0.002 or 0.005 mg mL^{-1}), the digestions were incubated at 37 °C for 1 h, an additional amount of thermolysin was added, and the digestion was continued for an additional hour. At least a 4-fold molar excess of phosphoramidone was then added to inhibit the thermolysin. Cyclization of amino-terminal glutamine residues was performed by addition of an equal amount of 0.5 M potassium phosphate, pH 8.0, and then incubation overnight at 37 °C (Gilbert et al., 1949). Pyroglutaminase digestions were carried out in 10 mM EDTA, 2.5 mM DTT, 5% glycerol, and 0.1 M sodium phosphate, pH 8.0. Pyroglutaminase (10 units) was added to a peptide in a minimal amount of buffer (less than 0.5 mL), the sample was flushed with argon, and the digest was incubated for at least 2 h at 37 °C. Digestions with leucine aminopeptidase (0.1 unit) were carried out in 2.5 mM $MgCl_2$, 0.1 M Tris−sulfate, pH 8.6 at 37 °C, for 4 h. Digestions with carboxypeptidase Y (5 units) were carried out in 0.1 M pyridinium acetate, pH 5.3, overnight at 37 °C.

Immunoadsorption. Digests were passed over the pyro-EQGM-immunoadsorbent 3 times. The unbound peptides were washed away with 7−10 column volumes of PBS, and the retained peptides were eluted with either 0.1 M sodium phosphate, pH 2.5, or 0.1 M glycinium hydrochloride, pH 2.5. The acid eluate was submitted to HPLC on a C-18 reverse phase column for further characterization. The immunoadsorbent was stored in PBS at 4 °C.

Analytical Methods. High-pressure liquid chromatography was performed on a system built from two Waters m6000A pumps, a Waters 680 automatic gradient controller, a Waters U6K injector, a Waters 440 absorbance detector fitted with an extended-wavelength module operating at 229 nm, and a Vydac C-18 reverse phase column (0.46 × 25 cm). Samples for amino acid analysis were hydrolyzed in 6 M HCl, under vacuum, for 40 min at 155 °C. Norleucine was added to each sample as an internal standard for estimating yield. Radioactivity was determined by dissolving samples in Ecolume cocktail and submitting them to liquid scintillation. Protein concentration was determined by the method of Lowry et al. (1951) or by amino acid analysis. Electrophoresis on 10% polyacrylamide gels in 0.1% SDS was performed as described by Laemmli (1970).

RESULTS

Preparation of an Immunoadsorbent Specific for Lysine 943. The first experiments were intended to determine if a small, water-soluble peptide containing the targeted lysine could be generated by proteolytic cleavage of the α polypeptide. The primary sequence of Na^+/K^+-transporting ATPase surrounding lysine 943 is -NSVFQQGMK(943)NKILIF-. From an inspection of the sequence, it appeared possible to generate the peptide QQGMK(943) by proteolytic digestion with trypsin and chymotrypsin or the peptide QQGMK(943)-NK by proteolytic digestion with chymotrypsin and thermolysin. The synthetic peptides pyro-EVFQQGMK and pyro-EQGMKNKILIF were synthesized manually by the solid phase FMOC method (Stewart & Young, 1984) to determine if indeed these specific cleavages would occur upon digestion and if the products would be water-soluble. Pyroglutamic acid was chosen as the amino-terminal residue so that each synthetic peptide would be blocked. Both peptides were shown to be homogeneous after purification by HPLC on a C-18 reverse phase column. The peptide pyro-EVFQQGMK eluted at 22% acetonitrile, and the peptide pyro-EQGMKNKILIF eluted at 34% acetonitrile. The peptide pyro-EVFQQGMK was submitted to acid hydrolysis and gave the composition $E_{3.0}V_{1.0}F_{1.0}G_{1.0}M_{1.0}K_{1.0}$. Carboxypeptidase Y released the expected amino acids in the ratio $K_{1.1}M_{1.1}G_{1.1}Q_{1.9}F_{1.1}V_{0.8}$; and, when the peptide was subjected to leucine aminopeptidase, no amino acids were liberated, consistent with a blocked amino terminus. Acid hydrolysis of the peptide pyro-EQGMKNKILIF gave the composition $E_{2.2}G_{1.0}M_{1.1}K_{2.2}D_{1.2}I_{1.5}L_{0.8}F_{1.0}$. Digestion of this peptide with leucine aminopeptidase produced no amino acids, consistent with a blocked amino terminus. A combination of carboxypeptidase Y, carboxypeptidase B, leucine aminopeptidase, and Pronase was used to digest this peptide and gave the composition $F_{1.0}I_{2.0}L_{1.0}K_{2.3}N_{0.8}G_{0.5}$.

When pyro-EVFQQGMK was digested with chymotrypsin, the peptides pyro-EVF and QQGMK were generated. The peptide QQGMK elutes by HPLC on a C-18 reverse phase column at 12% acetonitrile; and, upon cyclization of the amino-terminal glutamine to pyroglutamic acid, the

peptide shifts elution position to 14−15% acetonitrile. Thermolytic digestion of pyro-EQGMKNKILIF generated the peptide pyro-EQGMKNK, and digestion of the same peptide with trypsin generated the peptides pyro-EQGMK and ILIF. Both of the peptides that contained lysine, pyro-EQGMKNK and pyro-EQGMK, were water-soluble and were good candidates for immunoadsorption. The fact that digestion by chymotrypsin generated a peptide with an amino-terminal glutamine enabled advantage to be taken of the unique ability of glutamine to cyclize to pyroglutamic acid and create a blocked amino terminus. This simplified the production of antibodies against the amino-terminal sequence pyro-EQGM-.

Because both proteolytic digestions, either trypsin and chymotrypsin or thermolysin and chymotrypsin, produced peptides with the same amino-terminal sequence (pyro-EQGM-), this sequence was used to generate antibodies that would be capable of recognizing either peptide. The short peptide pyro-EQGMK was synthesized by the standard FMOC solid phase method. It was a homogeneous component eluting at 14−15% acetonitrile after isolation by HPLC on a C-18 reverse phase column. Acid hydrolysis gave the composition $E_{2.1}G_{1.0}M_{0.9}K_{1.0}$. Carboxypeptidase Y and pyroglutaminase followed by leucine aminopeptidase digestion gave the compositions $K_{1.0}M_{1.0}G_{0.4}$ and $Q_{1.2}G_{0.9}M_{0.9}K_{0.9}$, respectively. This synthetic peptide was conjugated to BSA and subsequently injected into rabbits; immunoglobulins were isolated; and an immunoadsorbent specific for pyro-EQGM- was prepared. The immunoadsorbent was initially capable of binding and releasing 13 nmol of synthetic pyro-EQGMK. The capacity of this immunoadsorbent was frequently monitored using the synthetic peptide pyro-EQGMK.

Isolation of an Indigenous Peptide by Immunoadsorption. The immunoadsorbent was used to isolate the peptide pyro-EQGMK from the α polypeptide of ovine Na^+/K^+-transporting ATPase. Microsomes rich in Na^+/K^+-transporting ATPase were dissolved with SDS and submitted to gel filtration in 0.1% SDS. Fractions containing the α polypeptide were pooled, and the SDS was removed from the polypeptide. Digestion with trypsin was followed by chymotrypsin, and amino-terminal glutamine residues were cyclized. The digest was then passed over the immunoadsorbent. All unbound peptides were washed off the column with PBS, and the retained peptides were eluted with acid. The acid eluate was submitted to HPLC on a C-18 reverse phase column. A peak of absorbance at 229 nm that eluted at 14−15% acetonitrile, the same elution position as the standard peptide pyro-EQGMK, was detected. This peak of absorbance was submitted to acid hydrolysis and gave the composition $E_{2.0}G_{1.1}M_{0.9}K_{0.9}$. This result verified that the correct peptide could be isolated from the α polypeptide of Na^+/K^+-transporting ATPase by the immunoadsorbent. In a typical experiment, 40 mg of microsomes (approximately 5 nmol of Na^+/K^+-transporting ATPase) would yield 2 nmol of isolated pyro-EQGMK.

Repeated attempts were made to isolate the longer peptide, pyro-EQGMKNK, from the α polypeptide by digestion with various combinations of chymotrypsin and thermolysin, but these were unsuccessful. Because the peptide pyro-EQGMK can be isolated readily from the α polypeptide, one can be confident that chymotrypsin has access and is capable of cleaving the peptide bond between phenylalanine 938 and glutamine 939. Because the synthetic peptide is cleanly cleaved by thermolysin, the inability to isolate the longer peptide suggests that thermolysin is not capable of cleaving the α polypeptide between lysine 945 and isoleucine 946 owing to the inaccessibility of this site to the proteinase.

Isolation of a Modified Peptide Containing Lysine 943. The only nucleophilic amino acids in close proximity to lysine 943 that have the potential of being modified are lysine 945 and lysine 943 itself. As noted previously, repeated attempts to isolate the peptide pyro-EQGMKNK that contains both of these lysines were unsuccessful, and, therefore, isolating this peptide from a thermolytic digest after one or the other of its lysines had been modified seemed unlikely. Another approach was taken.

Pyridoxal phosphate was chosen as an impermeant electrophile. It reacts with lysine residues to form an imine. This reaction was followed by reduction with sodium borohydride to form the stable secondary pyridoxamine. Using tritiated sodium borohydride enabled incorporation of radioactivity into the pyridoxal phosphate adducts with the lysine residues. From an inspection of the sequence surrounding lysine 943 (-NSVFQQGMKNKILIFGLFE-), the peptides that should be liberated after modification with a small amount of pyridoxal phosphate followed by digestion with chymotrypsin and trypsin are QQGMK and QQGMK-(Hpyr)NK, depending upon which lysine residue has been modified.

The synthetic peptide pyro-EQGMKNKILIF was modified with a high concentration of pyridoxal phosphate and sodium borohydride to generate the peptide pyro-EQGMK(Hpyr)-NK(Hpyr)ILIF. When this peptide was digested with trypsin, no cleavage occurred as expected. Thermolytic digestion gave the peptide pyro-EQGMK(Hpyr)NK(Hpyr) that eluted on HPLC on a C-18 reverse phase column at 14% acetonitrile. Acid hydrolysis gave the composition $E_{2.2}G_{1.1}M_{0.7}K_{0.1}D_{1.1}$.

The peptides pyro-EQGMK and pyro-EQGMK(Hpyr)NK-(Hpyr) coelute at 14−15% acetonitrile on HPLC. This observation suggests that modification of either lysine should lead ultimately to a peptide that will elute at 14−15% acetonitrile. Therefore, the singly modified peptide pyro-EQGMK(Hpyr)NK should coelute with the unmodified peptide pyro-EQGMK.

The immunoadsorbent specific for pyro-EQGM- was able to recognize and isolate the doubly modified peptide, but with a lower capacity compared to the unmodified peptide. This was determined by passing saturating amounts of the unlabled peptide pyro-EQGMK over the immunoadsorbent; approximately 2.5 nmol of peptide was bound and could be eluted. When saturating amounts of the modified peptide pyro-EQGMK(Hpyr)NK(Hpyr), however, were then passed over the same immunoadsorbent, only 0.5 nmol was retained and isolated. Although there was a difference in the capacity of the immunoadsorbent for the unmodified and modified peptides, this was not a concern because under the labeling conditions used in these studies (Kyte et al., 1987) less than 200 pmol of the modified peptide was ever produced. In addition, samples were always adjusted so that the total concentration of modified and unmodified peptide together was well below the capacity of the immunoadsorbent.

Topological Studies of Lysine 943 Using Sealed Vesicles. In order to determine the position of lysine 943 with respect to the plane of the bilayer, sealed vesicles were labeled with

the membrane-impermeant electrophile pyridoxal phosphate. The methods of Forbush (1982) as modified by Kyte et al. (1987) were used to prepare sealed, right-side-out vesicles from ovine renal medullas. Upon centrifugation, vesicles that are tightly sealed float upon a solution of the radioopaque dye sodium 3,5-bis(acetamido)-2,4,6-triiodobenzoate while unsealed vesicles sink. To determine if the vesicles are tightly sealed, a latency assay is performed by comparing the enzymatic activity of Na^+/K^+-transporting ATPase in these vesicles in the absence and the presence of a detergent. If the vesicles are tightly sealed, addition of substrates will not elicit Na^+/K^+-transporting ATPase activity since either the active site for MgATP or the site with which K^+ associates will lie on the inaccessible interior of the vesicle. When a detergent is added to sealed vesicles, the activity of Na^+/K^+-transporting ATPase dramatically increases as substrates gain access to the sites in the interior. By comparing the activities under each of these conditions, one can ascertain if the vesicles are tightly sealed.

Saponin, a heterogeneous mixture of glycosides that do no dissolve membranes readily but do produce holes of approximately 8 nm diameter (Bangam & Horne, 1962), was chosen for these studies. It had been shown previously (Kyte et al., 1987) that a saponin concentration of even 1.0% does not affect the enzymatic activity of Na^+/K^+-transporting ATPase. Saponin, unlike other detergents, does not denature the enzyme even at high concentrations, so one is more confident that the accessibility of buried amino acids to reagents will not be altered in vesicles treated with saponin and that saponin will only allow access of impermeant reagents to the cytoplasmic surface of the protein.

A fresh preparation of sealed vesicles (25−35 mg of protein) was split into two equal samples; one was treated with 0.4% saponin to breach the membranes, and the other was not. Both samples were then modified with 6 mM pyridoxal phosphate and sodium [^{3}H]borohydride. The α-polypeptide of Na^+/K^+-transporting ATPase from each sample was isolated by gel filtration in 1% SDS; the SDS was stripped from the polypeptide; proteolytic digestion of the polypeptide (at 1 mg mL^{-1}) was performed with trypsin followed by chymotrypsin, and the amino-terminal glutamine residues in the digest were cyclized. The digested and cyclized samples were each individually passed over the immunoadsorbent that recognizes the amino-terminal sequence pyro-EQGM-, and the retained peptides were eluted with acid. The acid eluates from the saponin-treated and the untreated vesicles were then separately subjected to HPLC on a C-18 reverse phase column, fractions were collected, and the radioactivity associated with each fraction was determined (Figure 1). In the chromatogram of the sample from the vesicles labeled in the absence of saponin (panel A), a peak of absorbance at 229 nm was detected at 14−15% acetonitrile, the same position at which the synthetic standard peptides pyro-EQGMK and pyro-EQGMK(Hpyr)-NK(Hpyr) elute during chromatography. The radioactivity associated with this peak was 62 cpm, and the height of the peak of absorbance was 9.3 cm. In the chromatogram of the sample from vesicles that were pretreated with 0.4% saponin before modification (panel B), a peak of absorbance was again detected at 14−15% acetonitrile, consistent with the elution position of the expected peptides. The radioactivity associated with this peak, however, was 1069 cpm

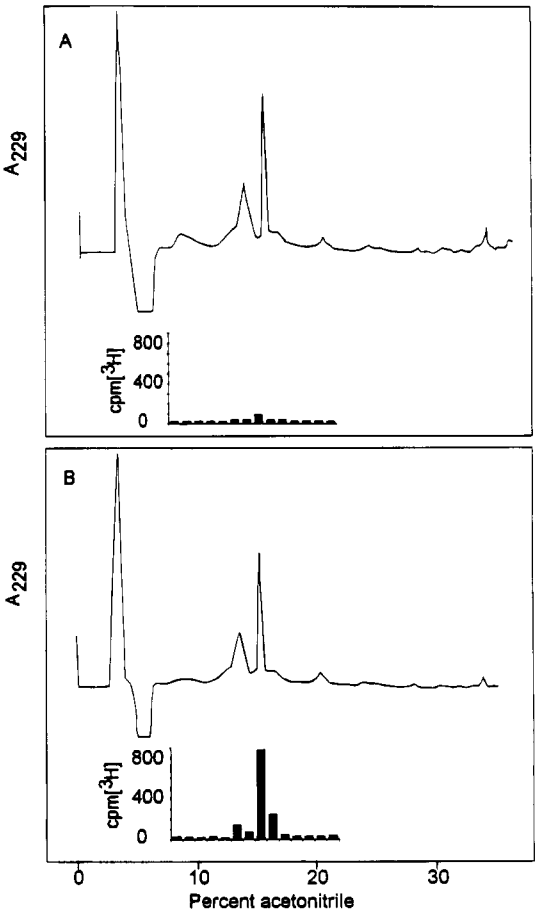

FIGURE 1: Incorporation of [^{3}H]pyridoxal phosphate into lysine 943 of the α polypeptide of Na^+/K^+-transporting ATPase in sealed, right-side-out vesicles. Two equal samples (25 mg of protein in 2.4 mL) of sealed, right-side-out vesicles were modified in the presence or absence of 0.4% saponin. After the addition of saponin to one sample, modification was carried out with 12 mM pyridoxal phosphate followed by 12.5 mCi (35 nmol) of [^{3}H]$NaBH_4$. The respective samples of isolated α polypeptide were digested with trypsin (0.3 mg 5 mL^{-1}) and chymotrypsin (0.3 mg 5 mL^{-1}) followed by cyclization (0.25 M potassium phosphate, pH 8.0 overnight at 37 °C). The digest was passed over an immunoadsorbent specific for pyro-EQGM- (capacity of 13 nmol). Unbound peptides were washed away with PBS, the bound peptides were eluted with 0.1 M glycine, pH 2.5, and the acid eluate was subjected to HPLC on a Vyadac C-18 reverse phase column (0.46 cm × 25 cm). A linear gradient was developed from 0% to 30% acetonitrile with solution A (0.05% TFA in H_2O) and solution B (0.017% TFA in acetonitrile) over 30 min at a flow rate of 1 mL min^{-1}. Absorbance was monitored continuously at 229 nm. The effluent was collected in 1 mL fractions and subjected to liquid scintillation counting to determine the amount of tritium. The inset displays the counts per minute of radioactivity in the fractions eluting between 8 and 21% acetonitrile, and the graph has been aligned with the high-pressure liquid chromatogram. (A) Results for the digest of samples from vesicles modified in the absence of saponin. (B) Results for the digest of samples from vesicles modified in the presence of saponin.

while the height of the peak was 9.0 cm. Consequently, pretreatment of the sealed vesicles with saponin increases the radioactivity associated with this peak 18-fold after correcting for the slight difference in the yield of peptide reflected in the difference in the height of the peaks of absorbance. In four separate labeling experiments with pyridoxal phosphate and sodium borohydride in the presence and absence of saponin, increases in the incorporation of radioactivity of 5−18-fold were observed (Table 1).

Table 1: Relative Specific Reactivity of Pyro-EQGMK([^{3}H]pyr)NK from Modified Vesicles[a]

		+saponin		−saponin		
expt	latency[c]	cpm[d]	peak height (cm)	cpm[d]	peak height (cm)	rel sp radioact.,[b] (cpm$^+$)(peak height$^-$)/ (cpm$^-$)(peak height$^+$)
		Sealed, Right-Side-Out Vesicles				
1	13	1069	9.0	62	9.3	18
2	10	1509	2.7	135	2.8	12
3	10	307	2.0	63	2.2	5
4	6	228	13.8	57	15.7	5
		Cholate Breached, Open Vesicles				
5	1	175	1.5	134	1.7	1.5
6	1	172	0.7	148	0.7	1.2

[a] The relative specific radioactivity of pyro-EQGMK([^{3}H]pyr)NK from modified vesicles was determined from experiments similar to that presented in Figure 1. Specific radioactivity is defined as the cpm observed divided by the height of the peak of absorbance at 14% acetonitrile. In this way the reactivity is normalized for the yield of peptides. [b] The relative specific radioactivity is the ratio of the specific radioactivity of samples from vesicles labeled in the presence of saponin to that of samples from vesicles labeled in its absence. [c] Latency is the ratio of enzymatic activity in vesicles in the presence of saponin compared to the enzymatic activity without treatment with saponin. High latency values indicate that the majority of vesicles are sealed. [d] Counts per minute above background in fractions at 14% acetonitrile.

Incorporation of Radioactivity in Breached Vesicles. In order to examine the question of whether or not the addition of saponin altered the accessibility of lysine 943 to pyridoxal phosphate by simply exposing buried regions of the folded polypeptide in the native protein, studies were performed with vesicles that had been breached with cholate prior to the addition of saponin. If it were the case that saponin treatment did indeed expose buried regions, one might expect to see an increase in incorporation of counts in vesicles breached with cholate and treated with saponin compared to vesicles breached with cholate but untreated with saponin prior to modification with pyridoxal phosphate. Sealed vesicles were titrated with increasing amounts of cholate to determine a concentration at which they were breached but the activity of Na^+/K^+-transporting ATPase did not drop due to denaturation induced by the detergent. Treatment with 1% cholate for 12 min was found to be sufficient to allow access for substrates to the cytoplasmic surface of the protein. These vesicles treated with cholate were split into two equal portions; 0.4% saponin was added to one portion and no saponin to the other. Modification with pyridoxal phosphate was then carried out; the α polypeptide was isolated and digested; and the peptides were cyclized, passed over the immunoadsorbent, and submitted to HPLC. In the sample from vesicles that had been treated only with cholate prior to modification, a peak of absorbance 1.7 cm in height was detected at 14−15% acetonitrile, and 134 cpm was associated with this peak (Table 1, experiment 5). Synthetic pyro-EQGMK was also run after this sample and eluted in the same position. In the sample from vesicles that had been treated with 1% cholate and then 0.4% saponin prior to modification, a peak of absorbance of 1.5 cm in height was detected and 175 cpm was associated with it. After correction for the slight difference in yield of peptide, a 1.5-fold increase in the incorporation of radioactivity into lysine 943 in saponin-treated over untreated vesicles was observed. The results of a second experiment (Table 1, experiment 6) with vesicles breached with cholate were the same.

Digestion of the Peptide Responsible for the Peak of Radioactivity with Pyroglutaminase. One limitation of using low levels of incorporation of a label into a peptide is that the labeled peptide cannot be isolated and submitted to amino acid analysis or sequencing to verify its identity. There is always a possibility that the radioactivity detected in the fractions from the HPLC is not associated with the expected peptide but some other peptide that happens to coelute at the same position as the desired peptide. In order to characterize the peak of radioactivity further, advantage was taken of the cyclized amino-terminal glutamine. Pyroglutaminase is an enzyme capable of cleaving amino-terminal pyroglutamic residues from peptides. Cleavage of an amino-terminal pyroglutamic acid residue from a peptide alters the elution position of that peptide on HPLC. Besides the fact that the peptide contains one fewer amino acid after digestion, it also has gained a charged amino terminus. Both of these consequences should cause the peptide to elute during HPLC at a lower concentration of acetonitrile. A portion of the sample from a control experiment in which the vesicles had been treated with cholate to expose both sides of the protein to modification by pyridoxal phosphate (Table 1, experiment 5) was reserved prior to immunoadsorption for digestion by pyroglutaminase. This portion was passed separately over the immunoadsorbent, eluted with acid, and submitted to HPLC (Figure 2A). Only one-fourth of each of the fractions was counted, and a small peak of radioactivity and absorbance at 229 nm was detected in the fraction eluting at 14−15% acetonitrile. The standard peptide pyro-EQGMK-(Hpyr)NK(Hpyr) was then run and was found to elute in the same position. The remaining three-fourths of only the one fraction containing both the radioactivity and the absorbance eluting at at 14−15% acetonitrile was dried down for digestion with pyroglutaminase. The standard peptide pyro-EQGMK(Hpyr)NK(Hpyr) was also digested to identify the position to which the radioactivity would be expected to move if it indeed belonged to pyro-EQGMK(Hpyr)NK. Initially, the peak of absorbance from the standard peptide and the radioactivity resided in the fraction eluting at 14−15% acetonitrile, but upon digestion with pyroglutaminase, the mobility of the standard peptide shifted to the fraction eluting at 12% acetonitrile. The radioactivity also shifted into the same fraction (Figure 2B). This was consistent with the assignment of the peak of radioactivity seen in all of these experiments at 14−15% acetonitrile as pyro-EQGMK-([^{3}H]pyr)NK. Although the peak of radioactivity at 14−15% acetonitrile displayed in panel A is quite small, all of the radioactivity displayed in panel B came from only this one fraction. The peak in the second chromatogram at 12% acetonitrile, which must represent radioactivity previously eluting at 14−15% acetonitrile, is well above background. It is highly unlikely that a modified peptide other than pyro-EQGMK(Hpyr)NK that possessed an amino-terminal glutamine residue capable of cyclization to pyroglutamic acid, that was recognized and retained by the immunoadsorbent, that eluted in the same position as pyro-EQGMK-(Hpyr)NK(Hpyr), and that when cleaved by pyroglutaminase moved to the same elution position as QGMK(Hpyr)NK-(Hpyr) would be generated from labeled Na^+/K^+-transporting ATPase by digestion with chymotrypsin and trypsin.

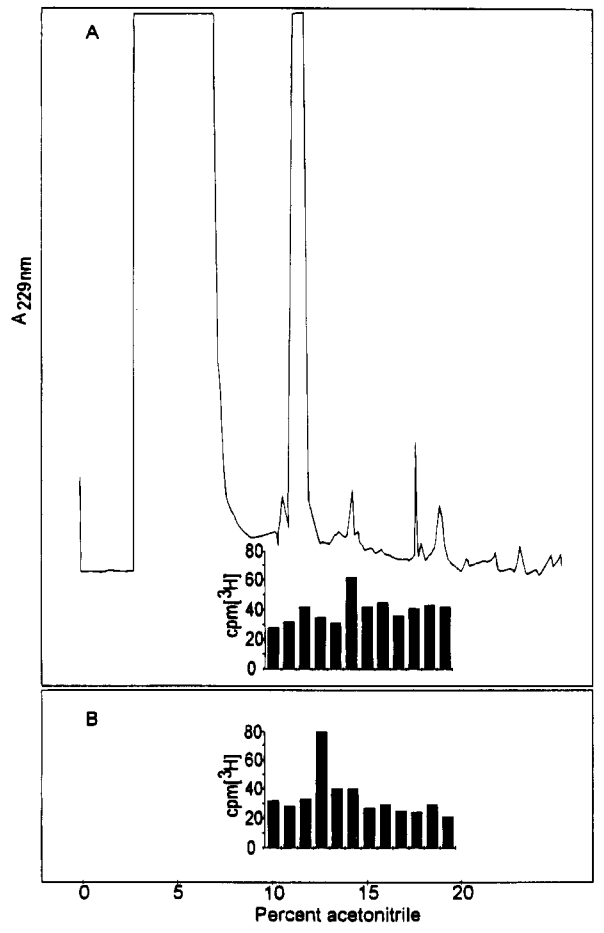

FIGURE 2: Digestion of pyro-EQGMK([^{3}H]pyr)NK with pyroglutaminase. (A) A sample of sealed, right-side-out vesicles that had been treated with cholate to open the vesicles prior to modification with pyridoxal phosphate was treated essentially as in Figure 1 with the following exceptions. The effluent from the HPLC was collected in fractions every 45 s, and, instead of counting the entire effluent from the HPLC, only one-fourth of each fraction was counted. The inset displays these counts per minute of radioactivity in the fractions eluting between 8 and 21% acetonitrile, and the graph has been aligned with the high-pressure liquid chromatogram. (B) The fraction containing the small peak of radioactivity and the peak of absorbance eluting at 14−15% acetonitrile was dried down, redissolved, digested with 10 units of pyroglutaminase for 2 h, and resubmitted to HPLC under the same conditions. The effluent was monitored at 229 nm. The entire contents of each fraction were subjected to liquid scintillation, and the inset displays the counts per minute of radioactivity associated with the fractions eluting between 8 and 21% acetonitrile. The graph had been aligned with the graph of panel A.

DISCUSSION

Ovine Na^+/K^+-transporting ATPase consists of two subunits, α and β. The β subunit contains 302 amino acids (Shull et al., 1986), spans the plasma membrane once, and is heavily glycosylated on the extracellular side of the membrane. No catalytic function has been assigned to the β subunit, but recently it has been shown that it is required to assemble and transfer the protein correctly from the endoplasmic reticulum to the plasma membrane (McDonough et al., 1990; Geering, 1990, 1991). The α subunit contains 1016 amino acids, spans the plasma membrane 4−10 times, and contains the site at which the phosphorylated intermediate in the reaction is formed and the active site for hydrolysis of MgATP and hence has been termed the catalytic subunit. It is the subunit of the present investigations.

In order to understand in detail the molecular mechanism of cation transport catalyzed by Na^+/K^+-transporting ATPase, knowledge of the structure of the α subunit is required. Because of the fact that all of the proteins in the family have evolved from the same common ancestor (Fagan & Saier, 1994), it follows that any structural information about one of these proteins would apply to the whole family. Integral membrane proteins are very difficult to crystallize, and only in a few examples have crystallographic molecular models at atomic resolution been obtained (Henderson et al., 1990; Deisenhofer et al., 1985; Weiss & Schulz, 1990, 1991, 1992; & Cowen et al., 1992). In the absence of crystallographic information, a variety of biochemical techniques have been developed to study the structural and functional relationships of membrane proteins.

The modification of sealed vesicles with small, water-soluble, impermeant electrophiles can be used to determine the topological disposition of particular amino acids with respect to the bilayer. Lysines are good candidates for this type of study since they are known to reside predominantly in positions on a protein accessible to the water when it is folded in its native conformation (Chothia, 1976). In the studies described here, lysine 943 was chosen because it lies between two putative membrane-spanning segments. Whether or not, and if so in which direction, the α polypeptide traverses the membrane in this region is still in debate. By using site-directed immunochemistry, which was first described by Kyte et al. (1987) and Bayer (1990) and subsequently refined by Dwyer (1991), Thibault (1993), and Ewalt (1994), I was able to establish that lysine 943 in the α subunit of Na^+/K^+-transporting ATPase occupies a cytoplasmic location.

In four separate experiments (Table 1, experiments 1−4), the ratio of radioactivity incorporated into lysine 943 from vesicles treated with saponin to that from untreated vesicles was assessed. In each experiment, after correction for yields of digestion, a ratio of 5 or higher was obtained, indicating that pyridoxal phosphate did not have access to lysine 943 unless the vesicles were breached by the addition of saponin prior to modification to allow the electrophile access to the cytoplasmic surface. Had the ratio of radioactivity in the saponin-treated and untreated vesicles been 1 or close to 1, it could have been concluded that the addition of saponin had no effect on the degree of modification of lysine 943 and that lysine 943 occupied an extracytoplasmic location.

In addition to disrupting biological membranes, surfactants can sometimes denature proteins. An even more serious concern in the present experiments is the denaturation of Na^+/K^+-transporting ATPase that occurs when a detergent strips it of its phospholipid (Kyte, 1972). Although Kyte et al. (1987) showed that saponin concentrations as high as 1% did not disrupt the enzymatic activity of Na^+/K^+-transporting ATPase, it had to be determined whether lysine 943 became more accessible to pyridoxal phosphate simply because of the addition of saponin. It was conceivable that saponin could loosen the native structure of the enzyme, exposing lysine 943, which would otherwise be buried, to pyridoxal phosphate. If this were the case, the high ratios of incorporation of radioactivity into vesicles treated with

saponin relative to the incorporation into untreated vesicles would lead one to conclude incorrectly that this residue resided cytoplasmically. In two separate control experiments (Table 1, experiments 5 and 6), an amount of cholate sufficient to open the vesicles but not affect the enzymatic activity was used. Following this addition of cholate, 0.4% saponin was either added or omitted. Because the vesicles were pretreated with cholate, pyridoxal phosphate had equal access to both sides of the lipid bilayer and was capable of modifying lysines on both surfaces of the protein. If addition of saponin had exposed lysine 943 by unfolding the protein, one would have expected to see a comparable increase in the incorporation of radioactive label. The results show that the labeling did not increase significantly upon addition of saponin to vesicles pretreated with cholate. From these results, it could be concluded that saponin itself has no direct effect on lysine 943 but that it acts solely to open the vesicles and expose the cytoplasmic surface to modification by pyridoxal phosphate.

To confirm that the radioactivity observed in the peak eluting at 14−15% acetonitrile belonged to lysine 943, digestion with pyroglutaminase was performed. The peak of radioactivity isolated behaved identically to the standard modified peptide pyro-EQGMMK(Hpy)NK(Hpyr). All of these experiments confirm that the modified peptide containing lysine 943 was isolated and properly identified and that this residue lies on the cytoplasmic surface of Na^+/K^+-transporting ATPase.

Recently Fisone et al. (1994) have shown that a serine residue in the rat kidney α 1 subunit of Na^+/K^+-transporting ATPase is a substrate for cAMP-dependent protein kinase A. Their studies suggest that serine 936 is the residue that is phosphorylated. Because it should be accessible for phosphorylation by intracellular cAMP-dependent protein kinase A, these results would assign a cytoplasmic location for this residue. The conclusions reached in these earlier experiments are in agreement with the conclusion that lysine 943 resides cytoplasmically.

Canfield and Levenson (1993), however, have recently reported results suggesting that amino acid 941 in the α subunit of rat Na^+/K^+-transporting ATPase occupies an extracellular location, which is in disagreement with my finding that lysine 943 resides cytoplasmically. Therefore it is necessary to discuss their experimental approach. Their strategy was to insert a immunoreactive, nine residue sequence of amino acides from the hemagglutinin of influenza virus into position 941 of the α subunit of Na^+/K^+-transporting ATPase and to express this mutated protein in human embryonic kidney 293 cells. Both permeablized (0.05% Nonidet P-40) and untreated cells were individually incubated with a hemagglutinin specific murine monoclonal immunoglobulin 12CA5 for 1h followed by Texas Red-conjugated rabbit anti-murine immunoglobulin. The cells were then viewed by fluorescence microscopy. In the case of the insertion mutant at position 941, a comparable amount of fluorescence was detected in both the permeabilized and untreated cells. Because treatment with 0.05% Nonidet P-40 did not increase binding of the primary immunoglobulin, the authors concluded that this region of Na^+/K^+-transporting ATPase has an extracellular location. In these experiments, the total concentration of Na^+/K^+-transporting ATPase in the cells relative to the amount of immunoglobulin bound was never assessed. Immunoglobulins are known to bind with a high affinity to denatured proteins, and a small amount of denatured protein always exists in any experimental preparation. Ideally, one would have to show that all of the Na^+/K^+-transporting ATPase in the preparation was capable of binding immunoglobulin from the extracellular surface. This result would rule out the possibility that it is only a small amount of denatured protein that is binding the primary immunoglobulin. This is particularly important in this instance because very little binding of the primary immunoglobulin is necessary to yield a positive result with a Texas Red conjugated secondary immunoglobulin. It should also be noted that it has been reported that antipeptide antibodies are capable of cross-reacting with other regions of a membrane-bound protein (Maelicke et al., 1989). In the experiments described in the present report, immunoglobulins were only used to isolate the appropriate peptide, not as probes of the native structure.

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REFERENCES

Argos, P., Rao, J. K. M., & Hargrave, P. A. (1982) *Eur. J. Biochem. 128*, 565−575.
Bangham, A. D., & Horne, R. W. (1962) *Nature (London) 196*, 952−953.
Bayer, R. (1990) *Biochemistry 29*, 2251−2256.
Bayley, H., & Knowles, J. R. (1980) *Biochemistry 19*, 3883−3892.
Brunner, J., & Semenza, G. (1981) *Biochemistry 20*, 7174−7182.
Burgess, R. R. (1969) *J. Biol. Chem. 244*, 6168−6176.
Canfield, V. A., & Levenson, R. (1993) *Biochemistry 32*, 13782−13786.
Chothia, C. (1976) *J. Mol. Biol. 105*, 1−14.
Cowan, S. W., Schrimer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., & Rosenbusch, J. P. (1992) *Nature (London) 358*, 727−733.
Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature (London) 318*, 618−624.
Dwyer, B. P. (1991) *Biochemistry 30*, 4105−4112.
Engelman, D. M., Steitz, T. A., & Goldman, A. (1986) *Annu. Rev. Biophys. Chem. 15*, 321−353.
Ewalt, K. L. (1994) *Biochemistry 33* 5077−5088.
Fagan, M. J., & Saier, M. H., Jr. (1994) *J. Mol. Evol. 38*, 57−99.
Fisone, G., Cheng, S. X.-J., Nairn, A. C., Czernik, A. J., Hemmings, H. C., Hoog, J.-O., Bertorello, A. M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., & Greengard, P. (1994) *J. Biol. Chem. 269*, 9368−9373.
Forbush, B. (1982) *J. Biol. Chem. 257*, 12678−12684.
Geering, K. (1990) *J. Membr. Biol. 115*, 109−121.
Geering, K. (1991) *FEBS Lett. 285*, 189−193.
Gilbert, J. B., Price, V. E., & Greenstein, J. P. (1949) *J. Biol. Chem. 180*, 209−218.
Guy, H. R. (1984) *Biophys. J. 45*, 249−261.
Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckman, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899−929.
Jørgensen, P. L., & Skou, J. C. (1969) *Biochem. Biophys. Res. Commun. 37*, 39−46.
Kuhn, L. A., & Leigh, J. S. (1985) *Biochim. Biophys. Acta 828*, 351−361.
Kyte, J. (1971) *J. Biol. Chem. 246*, 4157−4165.
Kyte, J. (1972) *J. Biol. Chem. 247*, 7642−7649.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105−132.
Kyte, J., Xu, K. Y., & Bayer, R. (1987) *Biochemistry 26*, 8350−8360.
Laemmli, U. K. (1970) *Nature (London) 227*, 680−691.

Lowry, O. H., Risebrough, N. J., Farr, A. L., & Randale, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Maelicke, A., Fels, G., Plumer-Wilk, R., Fels, G., Spencer, S. R., Engelhard, M., Veltel, D., & Conti-Tronconi, B. M. (1989) *Biochemistry 28*, 1396−1405.
McDonough, A., Geering, K., & Farley, R. A. (1990) *FASEB J. 4*, 1598−1605.
Nicholas, R. A. (1984) *Biochemistry 23*, 888−898.
Ohkawa, I., & Webster, R. E. (1981) *J. Biol. Chem. 56*, 9951−9958.
Rees, D. C., DeAntonio, L., & Eisenberg, D. (1989) *Science 245*, 510−513.
Shull, G. E., Lane, L. K., & Lingrell, J. B. (1986) *Nature (London) 321*, 429−431.
Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, IL.
Thibault, D. (1993) *Biochemistry 32*, 2813−2821.
Walter, G., Scheidtmann, K. H., Cargone, A., Laudano, A. P., & Doolittle, R. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5197−5200.
Weiss, M. S., & Schulz, G. E. (1990) *FEBS Lett. 267*, 268−272.
Weiss, M. S., & Schulz, G. E. (1991) *FEBS Lett. 280*, 379−382.
Weiss, M. S., & Schulz, G. E. (1992) *J. Biol. Chem. 227*, 493−509.

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