# Topological Disposition of the Sequences -QRKIVE- and -KETYY in Native $(Na^+ + K^+)$-ATPase†

Robert Bayer‡

*Department of Chemistry, University of California at San Diego, La Jolla, California 92093*

*Received June 28, 1989; Revised Manuscript Received October 27, 1989*

ABSTRACT: The dispositions with respect to the plane of the membrane of lysine-905 in the internal sequence -EQRKIVE- and of lysine-1012 in the carboxy-terminal sequence -RRPGGWVEKETYY of the α-polypeptide of sodium and potassium ion activated adenosinetriphosphatase have been determined. These lysines are found in peptides released from the intact α-polypeptide by the extracellular protease from *Staphylococcus aureus* strain V8 and by trypsin, respectively. Synthetic peptides containing terminal sequences of these were used to prepare polyclonal antibodies, which were then used to prepare immunoadsorbents directed against the respective peptides. Sealed, right-side-out membrane vesicles containing native $(Na^+ + K^+)$-ATPase were labeled with pyridoxal phosphate and sodium [$^3$H]borohydride in the absence or presence of saponin. The labeled α-polypeptide was isolated from these vesicles and digested with appropriate proteases. The incorporation of radioactivity into the peptides binding to the immunoadsorbent directed against the sequence pyrERXIVE increased 3-fold in the presence of saponin as a result of the increased accessibility of this portion of the protein to the reagent when the vesicles were breached by saponin; hence, this sequence is located on the cytoplasmic face of the membrane. It was inferred that the carboxy-terminal sequence -KETYY is on the extracytoplasmic face since the incorporation of radioactivity into peptides binding to the immunoadsorbent directed against the sequence -ETYY did not change when the vesicles were breached with saponin.

Sodium and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase][1] is the enzyme responsible for the vectorial transport of sodium and potassium ions across the plasma membrane of all animal cells. The enzyme consists of two subunits, designated α and β, found in equimolar ratio (Kyte, 1972; Liang & Winter, 1977; Craig & Kyte, 1980). The catalytic α-subunit spans the membrane (Kyte, 1975), and within it are located the binding site for ATP and the site that becomes phosphorylated during the catalytic cycle (Farley et al., 1984; Bastide et al., 1973). These sites are located on the cytoplasmic side of the membrane. The site to which cardiac glycosides bind, also located on the α-subunit, is on the extracytoplasmic side of the membrane (Ruoho & Kyte, 1974; Schwartz et al., 1975; Caldwell & Keynes, 1959).

The complete amino acid sequences from several species of the α-subunits have been deduced from the sequences of their complementary DNA (Shull et al., 1985, 1986; Kawakami et al., 1985; Herrera et al., 1987; Ovchinnikov et al., 1986). On the basis of computerized algorithms that identify candidates for membrane-spanning sequences on the basis of their hydropathy (Kyte & Doolittle, 1982; Engelman et al., 1986), 6–11 potential membrane-spanning segments have been designated within the sequence of the α-polypeptide, from which the α-subunit is formed. Alternatively, membrane-spanning segments of integral membrane proteins can be identified by labeling them chemically with hydrophobic, photogenerated nitrenes or carbenes (Bercovici & Gitler, 1978; Bayley & Knowles, 1980). Five radioactive tryptic peptides, presumably containing membrane-spanning segments, have been isolated and identified (Nicholas, 1984; Kyte, 1987) from α-polypeptide of canine renal $(Na^+ + K^+)$-ATPase after labeling the native enzyme with 1-tritiospiro[adamantane-4,3′-diazirine], a carbene precursor.

Some of the membrane-spanning segments predicted by the computational methods were not identified by the hydrophobic reagent, and the hydrophobic regions of three of the peptides identified by the hydrophobic reagent were not designated as candidates for spanning the membrane by the computational methods. Furthermore, since applications of the different computational methods to the amino acid sequence of the α-polypeptide do not designate the same set of membrane-spanning segments, the identity of the actual membrane-spanning segments and hence the folding pattern of the α-subunit across the plasma membrane remain to be resolved.

In the present study, the location of two hydrophilic regions of the α-subunit with respect to the plane of the membrane is established by chemical labeling of tightly sealed right-side-out vesicles containing $(Na^+ + K^+)$-ATPase with an impermeant reagent. Both the isolation and the identification of short peptides containing the labeled products were carried out by immunoadsorption. Control experiments with vesicles rendered permeable by saponin were used to identify those amino acids accessible only after the membrane had been breached. This approach has been used previously on the enzyme (Kyte et al., 1987) to demonstrate the cytoplasmic

†This research was supported by Grant GM-33962 from the National Institutes of Health, Grant AHA-870729 from the American Heart Association, and Grant DMB-8413772 from the National Science Foundation, all of which provide support to the laboratory of Dr. Jack Kyte. Postdoctoral Traineeships T32-AM07233-08 and 5-T32-AM07233-12 from the National Institutes of Health also provided support.

‡Present address: Pharmacia Genetic Engineering, 10280 N. Torrey Pines Rd., Suite 485, La Jolla, CA 92037.

[1] Abbreviations: $(Na^+ + K^+)$-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); Boc, *tert*-butoxycarbonyl; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; pyrE, pyroglutamate; phosphate-buffered saline, 135 mM sodium chloride, 0.1 mM EDTA, and 30 mM sodium phosphate, pH 7.4; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Affigel 10, agarose-activated with *N*-hydroxysuccinimide (Bio-Rad); Affigel–protein A, agarose to which protein A from *Staphylococcus aureus* has been covalently attached (Bio-Rad).

0006-2960/90/0429-2251$02.50/0 

disposition of lysine-501 in the α-subunit.

## Experimental Procedures

*Materials.* Pyridoxal phosphate, tris(hydroxymethyl)-aminomethane (Tris),[1] *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] saponin, histidine, soybean trypsin inhibitor, leucine aminopeptidase, carboxypeptidase B, $NaBH_4$, and 2-mercaptoethanol were purchased from Sigma Chemical Corp.; Sepharose 4B was from Pharmacia Corp.; Biogel A5M, succinylated agarose activated with *N*-hydroxysuccinimide (Affigel 10),[1] and agarose to which protein A from *Staphylococcus aureus* had been covalently attached (Affigel–protein A)[1] were from Bio-Rad Corp.; derivatives of amino acids protected at their α-amino nitrogens with *tert*-butoxycarbonyl (Boc)[1] groups were from Bachem Corp.; dicyclohexylcarbodiimide was from Aldrich Chemical Corp.; 1-deoxy-1-(methylammonium)-D-glucitol 3,4-diacetamido-2,4,6-triiodobenzoate (Hypaque meglumine as either a 50% or a 60% solution) was from Winthrop Breon Laboratories; trypsin that had been treated with L-(tosylamino)-2-phenylethyl chloromethyl ketone was from Worthington Corp.; ninhydrin and dimethyl pimelimidate were from Pierce Chemical Corp.; bovine serum albumin and the proteolytic enzyme from *Staphylococcus aureus* strain V8 were from Miles Diagnostics Corp.; Freund's adjuvant was from Difco Corp.; ammonium sulfate (enzyme grade) and urea (enzyme grade) were from Schwarz/Mann Corp. Sodium [$^3$H]borohydride was purchased as a solid from New England Nuclear Corp., and prepared as a solution at 50 mM in 10 mM NaOH and used immediately. Ovine kidneys were either obtained frozen from Pel-Freeze Corp. or obtained fresh from Superior Meat Packing Corp., Dixon, CA. Ecoscint and Ecolume scintillation cocktail were purchased from ICN Biomedicals. Solutions of formaldehyde were prepared from paraformaldehyde by heating.

*Preparation of ($Na^+$ + $K^+$)-ATPase and Sealed, Right-Side-Out Vesicles.* Purified ($Na^+$ + $K^+$)-ATPase in open fragments of membrane was prepared from either canine or ovine kidneys by the method of Jorgensen (1974) with the modifications described by Winslow (1981). Sealed, right-side-out vesicles, containing ($Na^+$ + $K^+$)-ATPase, were prepared from ovine or canine renal microsomes (Kyte, 1971) by flotation on gradients of Hypaque meglumine according to Forbush (1982). The increase in ($Na^+$ + $K^+$)-ATPase activity caused by addition of saponin to sealed, right-side-out vesicles was determined as described in Kyte et al. (1987).

*Protein Concentration, Amino Acid Analysis, and Electrophoresis.* Protein concentration in vesicles was estimated from $A_{280}$ in 2% sodium cholate; otherwise, quantitative amino acid analysis as described by Moczydlowski and Fortes (1981) was used. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Shapiro et al., 1967) was performed by the modifications of Weber and Osborn (1969). For scintillation counting, slices of polyacrylamide gels were prepared by the procedure of Drickamer (1976) as described by Munson (1983).

*Reductive Amination with Pyridoxal Phosphate and $NaB^3H_4$ and Isolation of Labeled α-Polypeptide.* The labeling procedure used was adapted from that of Ohkawa and Webster (1981). The buffer was prepared as follows: 60 mM boric acid was titrated with 60 mM NaOH to pH 9, the solution was then titrated to pH 8.0 with 60 mM phosphoric acid, and it was mixed with an equal volume of 0.5 M sucrose. Sealed, right-side-out vesicles dialyzed into this buffer were pretreated with 0.7 mM $NaBH_4$ for 10 min after addition of saponin to 0.2% to particular samples. Pyridoxal phosphate was then added to a final concentration of 6 mM. After 10 min, a half-molar equivalent of $NaB^3H_4$ was added to the solution, and the reduction was allowed to proceed for 15 min. All subsequent procedures were carried out in the dark. The samples were then dialyzed against four (24 h) changes of 0.25 M sucrose, 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetracetate (EDTA),[1] and 30 mM histidinium chloride, pH 7.4. The first change also included 1 mM pyridoxal phosphate and 10 mM lysine.

The modified, right-side-out vesicles were collected by centrifugation at 45 000 rpm for 90 min in a Beckman 50.2Ti rotor. The pellets were then taken up in dialysis buffer to which $NaDodSO_4$ had been added to 10%, and the solution was permitted to equilibrate at room temperature for at least 6 h. Modified α-polypeptide of ($Na^+$ + $K^+$)-ATPase was isolated from labeled vesicles by gel filtration on Biogel A5M in 0.1% $NaDodSO_4$/30 mM Tris–sulfate, pH 8.0. (Kyte, 1972). Fractions containing the α-polypeptide were identified by polyacrylamide gel electrophoresis, pooled, and lyophilized. The dodecyl sulfate was stripped from the polypeptides (Nicholas, 1984), and they were dialyzed into 50 mM ammonium bicarbonate buffer, pH 8.0.

*Proteolytic Digestions.* Tryptic digestions were carried out by adding trypsin at 1:10 by weight to the stripped α-polypeptide and incubating at 37 °C. After 6 h, an additional portion of trypsin was added, and the digestion was continued overnight. Soybean trypsin inhibitor (1:10 by weight) was then added. Chymotryptic and tryptic digestions also included chymotrypsin at a 1:10 weight ratio to substrates. Digestions with the proteolytic enzyme from *S. aureus* strain V8 were carried out by adding the proteolytic enzyme at a weight ratio of 1:7 and incubating at 37 °C. After 24 h, an equal volume of 0.5 M potassium phosphate, pH 8.0, was added and the incubation continued for another 24 h to cyclize amino-terminal glutamines to pyroglutamates (Gilbert et al., 1949).

*Peptide Synthesis.* The peptides pyrERKIVE (where pyrE[1] refers to pyroglutamate) and KETYY were synthesized manually by the method of Merrifield et al. (1982) using [[[4-(oxymethyl)phenyl]acetamido]methyl]poly(styrene-*co*-divinylbenzene) as the solid support. The following protected amino acids were used in the syntheses: $N^{\alpha}$-Boc-L-glutamic acid γ-benzyl ester, $N^{\alpha}$-Boc-L-valine, $N^{\alpha}$-Boc-L-isoleucine, $N^{\alpha}$-Boc-$N^{\epsilon}$-(benzoxycarbonyl)-L-lysine, $N^{\alpha}$-Boc-$N^{\delta}$-(*p*-toluenesulfonyl)-L-arginine, and pyro-L-glutamic acid to prepare pyrERKIVE; and $N^{\alpha}$-Boc-*O*-(dichlorobenzyl)-L-tyrosine (twice), $N^{\alpha}$-Boc-*O*-benzyl-L-threonine, $N^{\alpha}$-Boc-L-glutamic acid γ-benzyl ester, and $N^{\alpha}$-Boc-$N^{\epsilon}$-(benzoxycarbonyl)-L-lysine to prepare KETYY. The peptides were cleaved from the resin using liquid anhydrous HF, washed in anhydrous diethyl ether and ethyl acetate, extracted in 5% acetic acid in water, and lyophilized.

The peptides were purified by cation-exchange chromatography (Degen & Kyte, 1978), and additionally, for pyrERKIVE, by semipreparative high-pressure liquid chromatography (HPLC)[1] on a Waters μ-Bondapack C-18 column (7.8 × 300 mm) developed with a linear gradient between 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. Both peptides, following purification, displayed a single peak when submitted to analytical HPLC on a Waters μ-Bondapack C-18 column (3.9 × 300 mm) developed as described above.

*Preparation of Immunoadsorbents.* The peptides pyrERKIVE and KETYY were conjugated to bovine serum albumin through the amino groups of their lysines according to Walter et al. (1980). The peptides (10 mg) were dissolved in 2 mL

of 0.1 M sodium phosphate, pH 7.5, containing 20 mg of bovine serum albumin. Glutaraldehyde (1 mL of 20 mM) was added, and the reaction was carried out for 30 min at room temperature. The solution was then dialyzed against 135 mM sodium chloride, 0.1 mM EDTA, and 30 mM sodium phosphate, pH 7.4 (phosphate-buffered saline).[1]

These conjugates were then used to prepare polyclonal antibodies in white New Zealand rabbits. Initial injections in complete Freund's adjuvant (1:1 emulsion with 1 mg $mL^{-1}$ conjugate) were into the lymph nodes of the hind legs as well as intramuscularly in the back. Five weeks later, the rabbits were boosted subcutaneously in the back using an emulsion of the conjugates in incomplete Freund's adjuvant. Crude immunoglobulins were isolated from serum by precipitation in 50% saturated ammonium sulfate.

To purify immunoglobulins specific for the sequences pyrERXIVE and -ETYY, substituted agaroses were prepared for affinity chromatography by coupling the appropriate peptide to Affigel 10. Crude immunoglobulins precipitated with ammonium sulfate were dialyzed against phosphate-buffered saline and passed over the appropriate peptide affinity column. After the columns were extensively washed with phosphate-buffered saline, the bound antibodies were eluted with 0.1 M glycinium hydrochloride, pH 2.5, dialyzed against phosphate-buffered saline, and precipitated in 50% saturated ammonium sulfate.

These antibodies were then attached to agarose to produce immunoadsorbents. Purified immunoglobulins directed against the sequence pyrERXIVE (14.2 mg) were dialyzed against phosphate-buffered saline, and then 0.1 M HEPES, pH 7.45. These antibodies were then coupled to 3 mL of Affigel 10 by shaking overnight at 4 °C. Purified antibodies directed against the sequence -ETYY were cross-linked to Affigel–protein A by using dimethyl pimelimidate (Schneider et al., 1982).

For rapid determinations of the capacity of the immunoadsorbents, the synthetic peptides pyrERKIVE and KETYY were rendered radioactive by reductive methylation as described by Rice and Means (1971). Five milligrams of the peptide in 0.4 mL of 0.08 M formaldehyde/0.2 M sodium borate, pH 9.0, was treated with 0.06 mL of 0.13 M $NaB^3H_4$. After 60 min, 60 μL of 0.13 M $NaBH_4$ was added. After 60 min, the solution was dried under vacuum, and the radioactive peptides were purified by reverse-phase HPLC. The specific radioactivity of methylated pyrERKIVE was determined to be 14400 cpm $nmol^{-1}$; that of methylated KETYY, 13700 cpm $nmol^{-1}$.

*Immunoadsorption.* Digests of labeled protein were passed over the immunoadsorbent 3–5 times. The immunoadsorbent was then washed with phosphate-buffered saline. Bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5. Fractions (3 mL) were mixed with 12 mL of scintillant, and counted.

## Results

*Immunoadsorbent Specific for the Sequence pyrERXIVE.*[2] The synthetic peptide pyrERKIVE, after purification, behaved homogeneously upon submission to reverse-phase HPLC. Digestion with carboxypeptidase Y gave the following composition: $R_{0.74}K_{0.94}I_{1.00}V_{1.00}E_{1.00}$. Acid hydrolysis yielded 2.2 nmol of glutamic acid for every nanomole of peptide, calculated from the mean of the nanomoles of arginine and lysine. Amino acid analysis of the conjugate between pyrERKIVE and bovine serum albumin that was used to produce polyclonal antibodies specific for this sequence demonstrated that 6 nmol of peptide was coupled through its lysine to each nanomole of bovine serum albumin. Immunoglobulins directed against this conjugate were purified by affinity chromatography on a column of pyrERKIVE coupled to agarose through its lysine. The capacity of this column was estimated by digesting a sample of the resin with carboxypeptidase Y; 160 nmol each of valine and glutamic acid was released from every milliliter of the agarose.

The purified immunoglobulins were in turn coupled to agarose. The capacity of this immunoadsorbent for binding the synthetic peptide pyrERKIVE was determined to be 3.7 nmol of peptide (mL of agarose).$^{-1}$.

*Immunoadsorbent Specific for the Sequence -ETYY.* The synthetic peptide KETYY, after purification, behaved homogeneously upon submission to reverse-phase HPLC. Its amino acid composition after acid hydrolysis was $K_{0.95}E_{0.99}T_{1.18}Y_{1.88}$. Digestion with a combination of carboxypeptidase B and leucine aminopeptidase yielded the composition $K_{1.05}E_{0.87}T_{1.03}Y_{2.04}$. Amino acid analysis of the conjugate between KETYY and bovine serum albumin, that was used to produce the polyclonal antibodies, demonstrated that 20 nmol of the peptide was covalently attached through its lysine to each nanomole of bovine serum albumin. Immunoglobulins directed against this conjugate were purified by affinity chromatography on a column of KETYY attached to agarose through its lysine. Digestion of a sample of this agarose with carboxypeptidase Y released 610 nmol of tyrosine (mL of agarose)$^{-1}$.

An immunoadsorbent directed against the sequence -ETYY was then prepared by covalently attaching the affinity-purified immunoglobulins to Affigel–protein A. The capacity of the column, determined with [$^3$H]methyl-KETYY, was 5 nmol of peptide (mL of agarose)$^{-1}$.

*Determination of the Disposition of Lysine-905 and Lysine-1012 in Native $(Na^+ + K^+)$-ATPase.* The sealed, right-side-out vesicles (Forbush, 1982; Kyte et al., 1987) used in these experiments had $(Na^+ + K^+)$-ATPase activity that increased 5-fold in the presence of 0.2% saponin. This result demonstrates that at least 80% of the $(Na^+ + K^+)$-ATPase was present in vesicles impermeable to one or the other of the substrates that must gain access to the interior where the active sites of the enzyme are located and that these vesicles were rendered permeable by saponin. Sealed, right-side-out vesicles (72 mg in 18 mL) were divided into two equal portions. Saponin to 0.2% was added to one portion to render these vesicles permeable, and each sample was labeled with pyridoxal phosphate and $NaB^3H_4$. Equivalent samples of these vesicles were submitted to electrophoresis in 0.1% $NaDodSO_4$ on polyacrylamide gels, and the gels were sliced and submitted to scintillation counting (Kyte et al., 1987). Incorporation of tritium into the α-polypeptide of $(Na^+ + K^+)$-ATPase increased 1.5-fold in the presence of saponin. The modified α-polypeptide of $(Na^+ + K^+)$-ATPase was isolated from each sample by gel filtration in the presence of 0.1% $NaDodSO_4$, and the purified protein was then stripped of dodecyl sulfate.

For the analysis of the incorporation into lysine-905 in the sequence -EQRKIVE- within the sequence of native $(Na^+ + K^+)$-ATPase, the samples were digested with the proteolytic enzyme from *S. aureus* strain V8, and the amino-terminal glutamine residues of the resulting peptides were cyclized. Digests (6.3 nmol) were passed over a column of the immunoadsorbent (0.7 × 4 cm; 10-nmol capacity) directed against the sequence pyrERXIVE. After the immunoadsorbent was

[2] In this designation, X signifies the point of attachment of the hapten to the carrier, which should not contribute to the epitope.

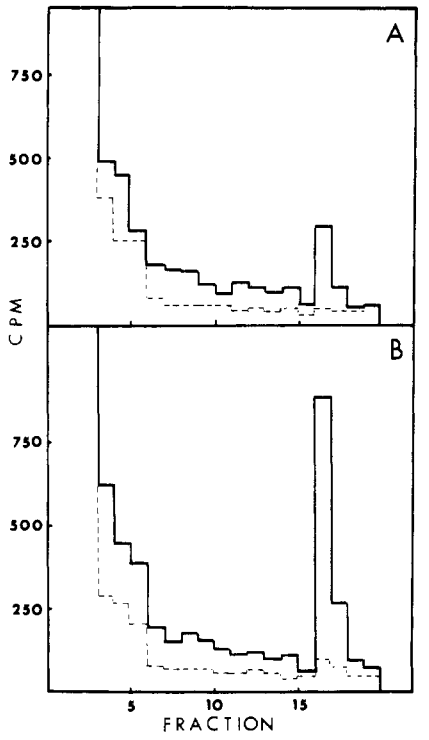


FIGURE 1: Labeling of lysine-905 in the α-subunit of ($Na^+$ + $K^+$)-ATPase. Labeled α-polypeptide (6.3 nmol) digested with the proteolytic enzyme from *S. aureus* strain V8 was added to a column (10-nmol capacity) of immunoadsorbent directed against the sequence pyrERXIVE. After the column was washed with phosphate-buffered saline (3-mL, fractions 1–15), bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5 (3-mL, fractions 16–20). (A) Digests of α-polypeptide from sealed, right-side-out vesicles labeled with pyridoxal phosphate and $NaB^3H_4$ in the absence of saponin (heavy line). (B) Digests of α-polypeptide from vesicles labeled in the presence of 0.2% saponin (heavy line). To demonstrate the specificity of the immunoadsorbent for the targeted sequence, an equivalent sample from each digest was mixed with 330 nmol of synthetic pyrERKIVE prior to application of the sample to the immunoadsorbent (dashed lines in panels A and B).

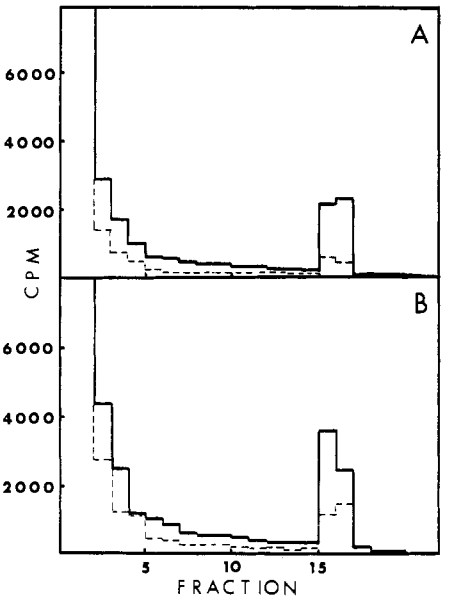


FIGURE 2: Labeling of lysine-1012 in the α-subunit of ($Na^+$ + $K^+$)-ATPase. Labeled α-polypeptide (13 nmol) digested with trypsin was applied to a column (20-nmol capacity) of immunoadsorbent directed against the carboxy-terminal sequence -ETYY. After the column was washed with phosphate-buffered saline (3 mL, fractions 1–15), bound peptides were eluted with 0.1 M sodium phosphate, pH 2.5 (3 mL, fractions 16–20). (A) Digests of α-polypeptide from vesicles labeled in the absence of saponin (heavy line). (B) Digests of α-polypeptide from sealed, right-side-out vesicles labeled with pyridoxal phosphate and $NaB^3H_4$ in the presence of 0.2% saponin (heavy line). To demonstrate the specificity of the immunoadsorbent for the targeted sequence, an equivalent sample from each digest was mixed with 230 nmol of synthetic KETYY prior to application of the sample to the immunoadsorbent (dashed lines in panels A and B).

washed with phosphate-buffered saline, bound peptides were eluted with acid, and their tritium content was determined by scintillation counting (Figure 1). As a control, to demonstrate the specificity of the immunoadsorbent for the peptide pyrERXIVE, an equivalent sample of each digest was mixed with a large excess (330 nmol) of the synthetic peptide pyrERKIVE prior to passage over the immunoadsorbent. This decreased the bound and eluted tritium significantly (Figure 1). In the case of α-polypeptide labeled in the presence of saponin (Figure 1A), 3-fold more tritium, in excess of control values, was eluted with acid than in the case of the α-polypeptide labeled in the absence of saponin (Figure 1).

For analysis of the incorporation into lysine-1012 in the sequence RRPGGWVEKETYY within the sequence of native ($Na^+$ + $K^+$)-ATPase, tryptic digests (13 nmol) were passed over a column of the immunoadsorbent (0.7 × 5 cm; 20-nmol capacity) directed against the sequence -ETYY. After the immunoadsorbent was washed, bound peptides were eluted with acid, and their tritium content was determined. Approximately the same number of counts per minute in excess of control values (3360 and 3410, respectively) were eluted for the sample from vesicles labeled in the presence of saponin (Figure 2A) as for the sample from vesicles labeled in the absence of saponin (Figure 2B).

An earlier set of experiments had been carried out exactly as the foregoing with the exception that the antibodies directed against the carboxy-terminal sequence -ETYY were not covalently attached to the Affigel–protein A, and were lost with each use. In these earlier experiments, incorporation of radioactivity into the peptides binding to the immunoglobulins directed against the sequence -ETYY increased 2-fold when the vesicles were breached with saponin, but the incorporation of radioactivity into the peptides binding to the immunoadsorbent directed against the sequence pyrERXIVE increased 4.9-fold.

## Discussion

To define the way in which a membrane-bound protein is folded across the membrane and to establish which putative membrane-spanning segments actually span the membrane, the disposition with respect to the plane of the membrane of the intervening hydrophilic regions among these hydrophobic segments can be determined. If one hydrophilic region flanking a potential membrane-spanning segment can be demonstrated to lie on the opposite side of the membrane from the other flanking region, then the intervening hydrophobic segment must span the membrane.

By comparing the level of modification of a target residue by an impermeant regent in both sealed and breached right-side-out vesicles, its disposition with respect to the plane of the membrane can be established. If the level of incorporation of the impermeant reagent increases significantly when the right-side-out vesicles are rendered permeable, then the targeted amino acid must lie on the cytoplasmic face of the membrane, which is directed toward the inside of a vesicle. In contrast, if the level of incorporation changes little when the vesicles are rendered permeable, the targeted amino acid must lie on the extracytoplasmic face of the membrane.

Potential membrane-spanning segments in the amino acid sequence of the α-subunit of ($Na^+$ + $K^+$)-ATPase (Figure 3)

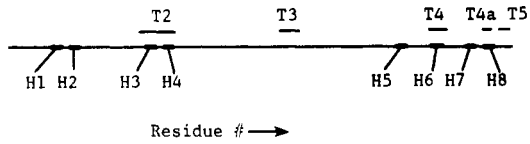


FIGURE 3: Distribution of potential membrane-spanning segments over the amino acid sequence of the α-polypeptide of $(Na^+ + K^+)$-ATPase. The locations are distributed over the line segment in proportion to their situation within the amino acid sequence of the α-polypeptide, and their lengths are drawn to scale. H1–H8: potential membrane-spanning sequences of α-polypeptide of $(Na^+ + K^+)$-ATPase identified by Shull et al. (1985) from plots of hydropathy. T2, T3, T4, T4a, and T5: tryptic peptides isolated from the α-polypeptide of canine renal $(Na^+ + K^+)$-ATPase labeled with 1-tritiospiro[adamantane-4,3′-diazirine] (Nicholas, 1984).

Table I: Hydropathy of Putative Membrane-Spanning Sequences of the α-Polypeptide of $(Na^+ + K^+)$-ATPase[a]

| putative membrane-spanning sequence | mean hydropathy[b] |
|---|---|
| H1 | 2.0 |
| H2 | 2.4 |
| H3 (included in T2) | 2.5 |
| H4 (included in T2) | 2.0 |
| T3 (residues 569–588) | 1.0 |
| H5 | 2.1 |
| H6 (included in T4) | 1.4 |
| H7 | 1.7 |
| H8 (included in T4a) | 1.6 |
| T5 (residues 973–993) | 1.0 |

[a] H1–H8 are potential membrane-spanning sequences identified by Shull et al. (1985) from plots of hydropathy. T2, T3, T4, T4a, and T5 are tryptic peptides from the α-polypeptide of canine renal $(Na^+ + K^+)$-ATPase labeled with 1-tritiospiro[adamantane-4,3′-diazirine] (Nicholas, 1984; Kyte, 1987). [b] Kyte and Doolittle (1982).

have been either identified by Shull et al. (1985) from hydropathy plots (H1–H8) or identified as tryptic peptides (T2, T3, T4, T4a, and T5) labeled by 1-tritiospiro[adamantane-4,3′-diazirine] (Nicholas, 1984; Kyte, 1987). Most established membrane-spanning sequences have a mean hydropathic index (Kyte & Doolittle, 1982) of 1.6 or greater, and this was the criterion used to designate potential membrane-spanning segments (Table I) in hydropathy plots. Proteins that form ion-carrying channels through a membrane, however, must necessarily contain polar residues within some of their membrane-spanning sequences, and this would lower the average hydropathy of those particular sequences. For example, the membrane-spanning segments of bacteriorhodopsin have mean hydropathic indexes ranging between 2.6 and 1.2 (Kyte & Doolittle, 1982).

Each of the first four hydrophobic, putative membrane-spanning segments in the amino acid sequence of the α-polypeptide, H1–H4, has a mean hydropathic index of 2.0 or greater. Within the large, predominantly hydrophilic region between H4 and H5 lie aspartate-369, the site phosphorylated by ATP (Bastide et al., 1972), and lysine-501, an amino acid residue in the active site at which ATP is hydrolyzed (Farley et al., 1984; Xu & Kyte, 1989). Both are known to lie on the cytoplasmic side of the membrane. The amino-terminal portion of the enzyme before H1 is also known to be exposed to the cytoplasmic side of the membrane (Castro & Farley, 1979; Karlish & Pick, 1981). Since there must be an even number of membrane-spanning segments between these two regions located on the cytoplasmic face of the membrane, and the segments, H1–H4, are so hydrophobic, it is likely that they all span the membrane. The intervening hydrophilic regions between H1 and H2, and between H3 and H4, contain no lysine residues, making this region of the protein unamenable to modification with the impermeant reagent, pyridoxal phosphate, used in this study. Therefore, the studies reported here have concentrated on the carboxy-terminal regions of the α-polypeptide.

Lysine-905 lies between segment T4 and segment H7 in the amino acid sequence of the α-polypeptide (Figure 3), and it is contained within the peptide QRKIVE released by digestion of the α-polypeptide by the proteolytic enzyme from *S. aureus* strain V8. The segment of amino acid sequence from which this peptide is derived lies between glutamate-902 and phenylalanine-909 in the amino acid sequence of the ovine α-polypeptide. In the experiment described in detail, incorporation of [$^3$H]pyridoxamine phosphate into lysine-905 increased 3-fold when sealed, right-side-out vesicles containing $(Na^+ + K^+)$-ATPase were rendered permeable by the addition of saponin (Figure 1). In the earlier experiment, the increase was 4.9-fold. Saponin has been shown to breach these vesicles without inactivating the enzyme (Kyte et al., 1987). This was also the case for the vesicles used in these studies. Because the enzyme is not inactivated by the surfactant, the only change that should be occurring is in the access of the reagent to the cytoplasmic surface of the enzyme. Therefore, these results demonstrate that lysine-905 lies on the cytoplasmic side of the membrane in native $(Na^+ + K^+)$-ATPase. Since the large hydrophilic sequence between H4 and T3 is also on the cytoplasmic side of the membrane, there must be an even number of membrane-spanning segments intervening. Hence, either two of the segments, T3, H5, and T4, must span the membrane, or none spans the membrane.

In the experiment described in detail, the level of incorporation of [$^3$H]pyridoxamine phosphate into lysine-1012 contained within the targeted tryptic peptide RPGGWVEKETYY, produced by digestion at arginine-1004 of the ovine sequence and including the carboxy-terminal sequence -ETYY, was not changed when the vesicles were rendered permeable by saponin (Figure 2). In the earlier experiment, an increase of 2-fold was observed, but this was at the same time that incorporation into lysine-905 increased 4.9-fold. It can be concluded from both the later and the earlier results together that lysine-1012 is located on the extracytoplasmic side of the membrane. Between lysine-905 and lysine-1012 there must be an odd number of membrane-spanning segments. In this region are found the three putative membrane-spanning segments: H7, H8 (T4a), and T5. Either only one or all three of these must span the membrane.

Another approach to determining the disposition of hydrophilic portions of a membrane protein with respect to the plane of the membrane has been to use antibodies as structural probes of the native protein. Equivocal results, however, have sometimes been obtained. The equivocation arises because the stoichiometry between the nanomoles of bound antibody and the nanomoles of the membrane-bound protein at saturation is usually very low, raising the possibility that the antibody is binding to a small, unrepresentative subpopulation of protein.

In the experiments described in this paper, only 1–2% of the lysines in the α-polypeptide are labeled, and this also raises the possibility that an unrepresentative population of the enzyme might be preferentially labeled. The degree of chemical modification is intentionally kept low to avoid possible structural alterations at high levels of modification (Bretscher, 1971). An examination of the incorporation of pyridoxal phosphate into the α-polypeptide of $(Na^+ + K^+)$-ATPase and into denatured bovine serum albumin, however, strongly suggested that under the conditions employed, the labeling reaction showed no preference for denatured protein (Kyte et al., 1987).

A proposal for the pattern in which the α-polypeptide folds across the membrane has been made on the basis of binding of antibody to embryonic porcine renal cells (Ovchinnikov et al., 1988). An increase in immunofluorescent staining of the cells upon treatment with 4% formaldehyde and 0.1% Tween 20 was observed. The monoclonal antibody used for this staining was found to bind peptides from a tryptic digest of porcine α-polypeptide corresponding to residues 881–904, which includes a portion of the amino acid sequence -QRKIVE- targeted in the present experiments. From these results, it is inferred that this region of the sequence was located on the cytoplasmic surface of the plasma membrane. In another experiment, polyclonal antibodies raised against a synthetic peptide corresponding to residues 999–1008 of the porcine α-polypeptide, which includes a portion of the targeted sequence PGGWVEKETYY, could elicit immunostaining of embryonic porcine renal cells that was 3–4-fold brighter than that in the controls to which the antibody was not added. It was inferred from these results that this region of the amino acid sequence was extracellular. Although the model resulting from these experiments agrees with the results obtained in the present study, caution should be exercised in interpreting the earlier observations due to the undetermined, but low, stoichiometry of binding observed and the indirect nature of the assay for binding of the antibody.

One of the purified immunoglobulins used in the present study, that directed against the carboxy-terminal sequence -ETYY, was found to bind to sealed, right-side-out vesicles as well as open membrane fragments of $(Na^+ + K^+)$-ATPase. The stoichiometry of binding at saturation, however, was determined to be less than 0.001 nmol of immunoglobulin (nmol of enzyme)$^{-1}$. Any conclusions about the native protein drawn from observations of the binding of this immunoglobulin would have been meaningless.

The method (Kyte et al., 1987) used in this study has a less stringent requirement for the region of the protein being studied: it must be accessible to a small chemical reagent rather than to the antigen binding site of an antibody. The present method also has its own limitations: there must be a reactive residue present in the region to be studied, a peptide containing the targeted residue should be readily produced by an appropriate proteolytic enzyme, and, lastly, the peptide so generated must be soluble in water.

ACKNOWLEDGMENTS

I thank Dr. Jack Kyte, in whose laboratory this research was performed, for his advice, support, and guidance.

REFERENCES

Bestide, F., Meissner, G., Fleischer, S., & Post, R. L. (1973) *J. Biol. Chem. 248*, 8385–8391.

Bayley, H., & Knowles, J. R. (1980) *Biochemistry 19*, 3883–2892.

Bercovici, T., & Gitler, C. (1978) *Biochemistry 17*, 1484–1489.

Bretscher, M. S. (1971) *J. Mol. Biol. 58*, 775–781.

Caldwell, P. D., & Keynes, R. D. (1959) *J. Physiol. 148*, 8P–9P.

Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221–2228.

Craig, W. S., & Kyte, J. (1980) *J. Biol. Chem. 255*, 6262–6269.

Degen, J., & Kyte, J. (1978) *Anal. Biochem. 89*, 529–539.

Drickamer, L. K. (1976) *J. Biol. Chem. 251*, 5115–5123.

Engelman, D. M., Steitz, T. A., & Goldman, A. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 321–353.

Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535.

Forbush, B. (1982) *J. Biol. Chem. 257*, 12678–12684.

Gilbert, J. B., Price, V. E., & Greenstein, J. P. (1949) *J. Biol. Chem. 180*, 209–218.

Herrera, V. L., Emanuel, J. R., Ruiz-Opazo, N., Levenson, R., & Nidal-Ginard, B. (1987) *J. Cell Biol. 105*, 1855–1865.

Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.

Karlish, S. J. D., & Pick, U. (1981) *J. Physiol. 312*, 505–529.

Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., & Numa, S. (1985) *Nature 316*, 733–736.

Kyte, J. (1971) *J. Biol. Chem. 246*, 4157–4165.

Kyte, J. (1972) *J. Biol. Chem. 247*, 7642–7649.

Kyte, J. (1975) *J. Biol. Chem. 250*, 7443–7449.

Kyte, J. (1987) in *Perspectives of Biological Energy Transduction* (Mukohota, Y., Morales, M. F., & Fleischer, S., Eds.) pp 231–239, Academic Press, Tokyo.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Kyte, J., Xu, K. Y., & Bayer, R. (1987) *Biochemistry 26*, 8350–8360.

Liang, S. M., & Winter, C. G. (1977) *J. Biol. Chem. 252*, 8278–8284.

Merrifield, R. B., Vizioli, L. D., & Boman, H. G. (1982) *Biochemistry 21*, 5020–5031.

Moczydlowski, E. G., & Fortes, P. A. G. (1981) *J. Biol. Chem. 256*, 2346–2356.

Munson, K. B. (1983) *Biochemistry 22*, 2301–2308.

Nicholas, R. A. (1984) *Biochemistry 23*, 888–898.

Ohkawa, I., & Webster, R. E. (1981) *J. Biol. Chem. 256*, 9951–9958.

Ovchinnikov, Yu. A., Modyanov, N. N., Broude, N. E., Petrukhin, K. E., Grishin, A. V., Arzamazova, N. M., Aldanova, N. A., Monastyrskaya, G. S., & Sverdlov, E. D. (1986) *FEBS Lett. 201*, 237–245.

Ovchinnikov, Yu. A., Luneva, N. M., Arystarkhova, E. A., Gevondyan, N. M., Arzamazova, N. M., Kozhich, A. T., Nesmeyanov, V. A., & Modyanov, N. N. (1988) *FEBS Lett. 227*, 230–234.

Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831–832.

Ruoho, A., & Kyte, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2352–2356.

Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U., & Greaves, M. F. (1982) *J. Biol. Chem. 257*, 10766–10769.

Schwartz, A., Lindenmeyer, G. E., & Allen, J. C. (1975) *Pharmacol. Rev. 27*, 3–134.

Shapiro, A. L., Viñuela, E., & Maizel, J. V., Jr. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.

Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691–695.

Walter, G., Scheidtmann, K. H., Carbone, A., Laudano, A. P., & Doolittle, R. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5197–5200.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Winslow, J. (1981) *J. Biol. Chem. 256*, 9522–9531.

Xu, K. Y., & Kyte, J. (1989) *Biochemistry 28*, 3009–3017.