The Carboxy Terminus of Sodium and Potassium Ion Transporting ATPase Is Located on the Cytoplasmic Surface of the Membrane†

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Received August 13, 1992; Revised Manuscript Received December 29, 1992

ABSTRACT: The positions, with respect to the plasma membrane, of lysine 905, contained in the peptide QRKIVE, and of lysine 1012, contained in the carboxy-terminal peptide, RPGGWVEKETYY, of ovine Na^+/K^+-transporting ATPase have been reported to be cytoplasmic and extracytoplasmic, respectively [Bayer, R. (1990) *Biochemistry 29,* 2551–2256]. These results from our laboratory have been reexamined using an extension of the same procedure. Sealed right-side-out vesicles were modified with pyridoxal phosphate and sodium [^{3}H]borohydride in the presence and absence of saponin or cholate. The modified α polypeptide was isolated and digested with the proteinase from *Staphylococcus aureus* strain V8 or trypsin to produce one or the other of these two peptides. These digests were passed over immunoadsorbents, identical to those used by Bayer, directed against pyroglutamylRXIVE or -ETYY. Unlike in the earlier studies, however, in the present studies the modified, radioactive peptides bound and eluted from the immunoadsorbents were submitted to HPLC, and their respective mobilities were compared to those of the synthetic peptides that had also been modified with pyridoxal phosphate. In this manner, the correct, modified peptide could be positively identified, and its specific radioactivity could be estimated. When cholate was added to sealed vesicles, prior to modification, there was at least a 3-fold increase in the incorporation of radioactivity into lysine 1012, consistent with a cytoplasmic location for this residue. It was not possible to determine the position of lysine 905 owing to the difficulty in modifying this residue with pyridoxal phosphate, but it could be shown that the conclusions of Bayer (1990) concerning this peptide were compromised by the presence of a contaminating, modified peptide, RYAK(^{3}Hpyr)IVE.

Na^+/K^+-transporting ATPase [(Na^++K^+)-ATPase][1] from animal plasma membranes (EC 3.6.1.3) is a member of the E_1E_2 superfamily of cation-transporting ATPases, which includes endoplasmic reticular Ca^{2+}-transporting ATPase, calmodulin-activated, plasma membrane Ca^{2+}-transporting ATPase, gastric mucosal H^+/K^+-transporting ATPase, fungal H^+-transporting ATPase, and plant H^+-transporting ATPase. Members of this superfamily are known to have descended from a common ancestor and therefore must be similar in their kinetic mechanisms, secondary, tertiary, and quaternary structures, and dispositions in the lipid bilayer (Kyte, 1981). Na^+/K^+-transporting ATPase consists of one α subunit and one β subunit (Craig, 1982). The β subunit seems to be unique to (Na^++K^+)-ATPase and gastric mucosal H^+/K^+-transporting ATPase (Okamoto et al., 1990; Reuben et al., 1990; Shull, 1990; Toh et al., 1990). It is a glycoprotein with no known function. The α subunit is composed of the folded polypeptide homologous to that of the other transporting ATPases. It is responsible for the catalytic activity and is known to span the membrane (Ruoho & Kyte, 1974). It contains the binding site for MgATP (Farley et al., 1984) and the aspartate that is phosphorylated during turnover (Bastide et al., 1973; Post, 1973); both are located on the cytoplasmic surface of the enzyme. The amino acid sequences forming the binding site for cardiac glycosides are on the extracytoplasmic surface of the α subunit (Ruoho & Kyte, 1974; Price & Lingrel, 1988).

To understand the mechanism of cation transport, it will be necessary to determine which of the segments of amino acid sequence span the membrane and form the channel through which cations pass. One approach to this problem has involved the use of hydrophobic reagents, which partition into the membrane, to modify the membrane-spanning segments. Using this method, Nicholas (1984) identified five tryptic peptides modified with 1-tritiospiro(adamantane-4,3′-diaziridine).

Alternatively, computer algorithms, based on the hydropathies of the amino acids, have been used to predict membrane-spanning segments in the amino acid sequences of proteins (Kyte & Doolittle, 1982; Eisenberg et al., 1984; Engelman et al., 1986). The amino acid sequences of the α subunit from a number of species have been determined (Shull et al., 1985; Kawakami et al., 1985; Herrera et al., 1987; Ovichinnikov et al., 1986), and these algorithms were immediately applied to these sequences. These calculations and similar ones applied to the amino acid sequences of other members of the superfamily led to the predictions that six to ten segments of the α polypeptide spanned the membrane (Kawakami et al., 1985; Ovchinnikov et al., 1987; Shull & Greeb, 1988; Shull et al., 1985; Brandel et al., 1986; Serrano, 1988; MacLennan et al., 1985; Mandala & Slayman, 1989). The experimental and computational results are not in complete agreement; and, at this time, the identities of the membrane-spanning segments are uncertain.

† This research was supported by Grant GM-33962 from the National Institutes of Health, which provides support to the laboratory of Dr. Jack Kyte, and Training Grant GM-07313 from the National Institutes of Health.

[1] Abbreviations: (Na^++K^+)-ATPase, Na^+/K^+-transporting adenosinetriphosphatase (EC 3.6.1.3); meglumine ditrizoate, the 1-deoxy-1-(methylammonium)-D-glucitol salt of 3,5-bis(acetylamino)-2,4,6-triiodobenzoate; sodium ditrizoate, the sodium salt of 3,5-bis(acetylamino)-2,4,6-triiodobenzoate; Fmoc, 9-fluorenylmethoxycarbonyl; Wang's resin, *p*-alkoxybenzyl alcohol resin; $NaDodSO_4$, sodium dodecyl sulfate; pyro-E, pyroglutamyl; Tris, tris(hydroxymethyl)aminomethane; phosphate-buffered saline, 150 mM sodium chloride, 20 mM sodium phosphate, 0.1 mM ethylenediaminetetraacetate, pH 7.4; HPLC, high-pressure liquid chromatography; C_{18}, octadecylsilyl-silica; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; K(^{3}Hpyr), lysine modified by pyridoxal phosphate and sodium [^{3}H]borohydride; Na[^{3}H]BH_4, sodium [^{3}H]-borohydride.

0006-2960/93/0432-2813$04.00/0

Another approach to this problem focuses on hydrophilic regions on either side of a potential membrane-spanning segment. If two consecutive, hydrophilic regions are demonstrated to lie on opposite faces of the membrane, then the hydrophobic segment between them must span the bilayer. Immunoglobulins directed against particular hydrophilic regions have been used in experiments with various ATPases in their native structures to define the locations of the epitopes relative to the plane of the bilayer, but the reliability of this method is uncertain (McCrea et al., 1987; Maelicke et al., 1989). Kyte et al. (1987) modified the hydrophilic regions of $(Na^+ + K^+)$-ATPase in sealed vesicles, with the impermeant reagent, pyridoxal phosphate, in the presence and absence of the glycoside saponin. After enzymatic digestion, one specific peptide that had been selected as a target was isolated with an immunoadsorbent. This procedure demonstrated that lysine 501 was on the cytoplasmic surface of the membrane (Kyte et al., 1987). This approach has been used by Dwyer (1991) to show that two lysines on either side of a membrane-spanning segment of acetylcholine receptor were on opposite sides of the membrane.

This approach was also used in a report from our laboratory by Bayer (1990) in which it was concluded that the locations of lysine 905 and lysine 1012 were cytoplasmic and extracytoplasmic, respectively. Unfortunately, a number of groups have reported the opposite locations for these regions in endoplasmic reticular Ca^{2+}-transporting ATPase, calmodulin-activated, plasma membrane Ca^{2+}-transporting ATPase, and fungal H^+-transporting ATPase (James et al., 1988; Mandala & Slayman, 1989; Hennessey & Scarborough, 1990; Clarke et al., 1990; Matthews et al., 1989, 1990). In this report, a further elaboration of the technique used by Kyte et al. (1987) and Dwyer (1991) has been used to reexamine the location of these two regions in $(Na^+ + K^+)$-ATPase. The results reported here demonstrate that the conclusions reached by Bayer (1990) were in error.

EXPERIMENTAL PROCEDURES

Materials. Pyridoxal phosphate, adenosine 5′-triphosphate, N^{α}-acetyl-L-arginine, strophanthidin, thioanisole, Amberlite XAD-4, the 1-deoxy-1-(methylammonium)-D-glucitol and sodium salts of 3,5-bis(acetylamino)-2,4,6-triiodobenzoate (ditrizoate),[1] phosphoramidone, soy bean trypsin inhibitor, methylene blue, sodium borohydride, and sodium cyanoborohydride were purchased from Sigma Chemical Co.; 1-hydroxybenzotriazole hydrate, 1,3-diisopropylcarbodiimide, and 1,2-ethanedithiol were purchased from Aldrich Chemical Co.; acetonitrile (high-pressure liquid chromatography grade) was purchased from Fisher; and Bio-Gel A-5M, Bio-Gel A-1.5M, and Dowex AG 1X2 resin were purchased from Bio-Rad Corp. The otherwise blocked amino acids with 9-fluorenylmethoxycarbonyl protecting groups (Fmoc)[1] on their α amino groups were purchased from Beckman and Bachem Corp.; *p*-alkoxybenzyl alcohol resin (Wang's Resin)[1] was purchased from Bachem Corp.; carboxypeptidase Y, saponin, and thermolysin were purchased from Calbiochem Corp.; trypsin that had been treated with *N*-(*p*-toluenesulfonyl)-L-phenylalanyl chloromethyl ketone and the proteinase from *Staphylococcus aureus* strain V8 were purchased from Worthington Corp.; and ultrapure urea was purchased from ICN Corp. Ovine kidneys were purchased fresh from Superior Meat Packing Corp., Dixon, CA. Sodium [^{3}H]borohydride (490 mCi mmol^{-1}) was purchased as a solid from New England Nuclear Corp. It was dissolved in 0.2 mL of 0.01 M NaOH and used immediately. Imidazole was purchased from Sigma Chemical Co. and recrystallized from benzene and then from acetone. Sodium dodecyl sulfate ($NaDodSO_4$)[1] was purchased from Sigma Chemical Co. or Calbiochem Corp. and recrystallized from 95% ethanol (Burgess, 1969). Cholate was purchased from Sigma Chemical Co. and prepared by boiling with Norit A in 95% ethanol, filtering, and recrystallizing from 70% ethanol.

Synthesis of Peptides. The synthesis and purification of the peptides pyroERKIVE and KETYY were described previously in Bayer (1990). The peptide *N*-acetylRRPGGWVEKETYY was synthesized using the Fmoc method (Stewart & Young, 1984) with *p*-alkoxybenzyl alcohol resin (Wang's resin) as the solid support. The following protected L-amino acids were used: N^{α}-Fmoc-*O*-butyl-L-tyrosine, N^{α}-Fmoc-O^{β}-*tert*-butyl-L-threonine, N^{α}-Fmoc-L-glutamic acid γ-butyl ester, N^{α}-Fmoc-N-ϵ-(*tert*-butyloxycarbonyl)-L-lysine, N^{α}-Fmoc-L-valine, N^{α}-Fmoc-L-tryptophan, N^{α}-Fmoc-L-glycine, N^{α}-Fmoc-L-proline, N^{α}-Fmoc-N^{δ}-4-methoxy-2,3,6-trimethylbenzenesulfonyl-L-arginine. N^{α}-Acetyl-L-arginine was used to block the amino terminus of the peptide to prevent its modification. After the amino terminus was deblocked at the last cycle, *N*-acetyl-L-arginine (0.185 M) in dimethyl sulfoxide was added. The coupling was performed with diisopropylcarbodiimide (0.185 M) for 120 min at room temperature. The peptide was cleaved from the resin using 71% trifluoroacetic acid and 12% trimethylsilyl bromide, 5% ethanedithiol, 1% *m*-cresol, and 11% thioanisole as scavengers (Gou et al., 1988).

Preparation of Right-Side-Out, Sealed Vesicles and Open Fragments of Membranes. Ovine, renal microsomes were prepared according to Kyte (1971) and floated on a gradient of sodium ditrizoate by the modification of the procedure of Forbush (1982) described by Dwyer (1991). The bands of vesicles that floated were collected, diluted with 250 mM sucrose, 30 mM sodium borate, 30 mM imidazolium chloride, pH 8, and pelleted at 40 000 rpm in a Beckman Ti 45 rotor for 1 h. The vesicles were then resuspended and homogenized gently in a minimal amount of the same buffer.

Open fragments of membrane were obtained by collecting the material that had pelleted at the bottom of the gradient of sodium ditrizoate. These membranes were diluted with the same buffer and pelleted at 40 000 rpm in a Beckman Ti 45 rotor for 1 h. The pellet was resuspended and repelleted to remove the sodium ditrizoate. The final pellet was resuspended in a minimal amount of buffer.

Both sealed vesicles and open fragments of membrane were assayed for $(Na^+ + K^+)$-ATPase to determine their latency. Latency is defined as the increase in strophanthidin-sensitive enzymatic activity upon addition of saponin (Kyte, 1971; Kyte et al., 1987). Sealed vesicles were used immediately; open fragments could be stored at −70 °C prior to use.

Labeling of Sealed Vesicles and Open Fragments of Membranes and Isolation of the α Polypeptides. Sealed vesicles or open fragments of membrane in a minimal volume of 250 mM sucrose, 30 mM sodium borate, 30 mM imidazolium chloride, pH 8, were divided in two. Saponin was added to a concentration of 0.4% or cholate was added to a concentration of 1.2% to half, and the samples were stirred for 12 min. Both halves were then treated with 0.7 mM sodium borohydride for 10 min, after which pyridoxal phosphate was added to 12 mM and the mixture was stirred for 15 min. Sodium [^{3}H]borohydride (12.5 mCi) was added to each sample. After 20 min, either a 5-fold [5 g (g of protein)$^{-1}$] excess of $NaDodSO_4$ was added or the samples were diluted and pelleted in a Beckman Ti 45 rotor at 40 000 rpm for 1 h. In the latter instances, the pellets were resuspended and

a 5-fold excess [5 g (g of protein)$^{-1}$] of $NaDodSO_4$ was added. The former method resulted in higher yields of protein. In both cases, the samples were stirred for at least 1 h at room temperature after $NaDodSO_4$ was added and before the samples were applied to columns of either Bio-Gel A-5M or Bio-Gel A-1.5M in 0.04 M Tris–sulfate, 0.1% $NaDodSO_4$, pH 8. Fractions of the eluate from the columns were screened for the α polypeptide of $(Na^+ + K^+)$-ATPase by electrophoresis on gels of 10% polyacrylamide cast in 0.1% $NaDodSO_4$. The fractions containing the α polypeptide were pooled and lyophilized. The lyophilized material was dissolved in a minimal amount of water, urea was added to 8 M, and the $NaDodSO_4$ was removed by the method of Weber and Kuter (1971) as modified by Nicholas (1984). The urea was removed by dialysis against 0.1 M ammonium bicarbonate. All procedures involving pyridoxal phosphate were performed in dim light.

Proteolytic Digestions. Tryptic digestions were performed in 0.1 M ammonium bicarbonate, 0.1 mM calcium chloride, pH 8. Trypsin was added at a ratio of 1:100 with respect to the amount of protein, and the samples were incubated at 37 °C. After 1 h an equal amount of trypsin was added. In one more hour, a 4-fold excess [4 g (g of trypsin)$^{-1}$] of soy bean trypsin inhibitor was added. Digestions with the proteinase from *S. aureus* strain V8 were performed in 0.1 M ammonium bicarbonate, pH 7.8. The proteinase was added to a concentration of 2% by weight of protein, and the samples were incubated at 37 °C for 2 h. To cyclize the glutamines in relevant samples, an equivalent volume of 0.5 M potassium phosphate, pH 8, was added and the samples were incubated at 37 °C for 20–24 h (Gilbert et al., 1949). Digestions with thermolysin were performed in 0.1 M ammonium bicarbonate, 1 mM calcium chloride. Thermolysin was added to 0.01% (0.1 μg mL^{-1}) and the samples were incubated at 37 °C for 1 h and then inhibited with a 10 molar excess of phosphoramidone. Digestions with carboxypeptidase Y were performed in 0.1 M pyridinium acetate, pH 5.5. Five units (approximately 30 μg) of the enzyme were added to the substrate (usually about 5 nmol), and the sample was incubated overnight at 37 °C. The extent of digestion was quantified by submitting the samples to amino acid analysis.

Immunoadsorption and High-Pressure Liquid Chromatography. The immunoadsorbents used in these studies were those prepared in our laboratory by Bayer (1990). The digested, modified α polypeptide was passed over an immunoadsorbent three times. The immunoadsorbent was washed with phosphate-buffered saline, and the bound peptides were eluted with 1 M acetic acid. These acid eluates were then submitted to reverse-phase, high-pressure liquid chromatography (HPLC).[1] The gradient for HPLC was developed with increasing amounts of acetonitrile containing 0.017% TFA at a rate of 1% min^{-1} after equilibration in 0.05% TFA in water. One of two Vydac C_{18} columns was used, an analytical column (0.46 × 25 cm) or a semipreparative column (2.2 × 25 cm). The system for HPLC consisted of a Waters UK6 injector, two Waters M6000 pumps, a Waters 440 detector modified for operation at 229 nm, and a Waters 680 gradient controller.

Modification of N-AcetylRRPGGWVEKETYY with Pyridoxal Phosphate. Pyridoxal phosphate was dissolved in 0.1 M sodium phosphate, pH 8, to make a saturated solution (~1.85 M). The pH was readjusted with 12 M NaOH to pH 8. This saturated solution was added to the solid peptide *N*-acetylRRPGGWVEKETYY and diluted so that the final concentrations of pyridoxal phosphate and peptide were 1.5 M and 5 mM, respectively. After 20 min, a 2 molar equivalent of sodium cyanoborohydride was added. The mixture was stirred for 20 min, and then acetic acid was used to reduce the pH. The modified peptide was purified from the labeling reagents on reverse-phase HPLC. The peptide was digested with trypsin to remove the *N*-acetylarginine and repurified on HPLC.

Protein Concentration, Amino Acid Analysis, Determination of Radioactivity, and Peptide Sequences. Protein concentrations were determined by the method of Lowry et al. (1951). Samples for amino acid analysis were submitted to acid hydrolysis by heating in 6 M HCl under vacuum for 40 min at 155 °C. Radioactivity was determined by dissolving samples in Ecolume (ICN Corp.) and submitting them to liquid scintillation. Peptides were sequenced on a Applied Biosystems 470 protein sequencer with an on-line PTH Applied Biosystems 120A analyzer.

RESULTS

Modification of Synthetic N-AcetylRRPGGWVEKETYY with Pyridoxal Phosphate. The peptide *N*-acetylRRPGGWVEKETYY was synthesized to use as an experimental standard for the tryptic peptide RPGGWVEK(^{3}Hpyr)ETYY [where K(^{3}Hpyr) indicates lysine modified by pyridoxal phosphate and [^{3}H]borohydride].[1] This is the peptide that should be released from the carboxy terminus of the modified α polypeptide of $(Na^+ + K^+)$-ATPase by tryptic digestion. The crude peptide was submitted to HPLC, and two closely spaced peaks were observed. They were shown to have indistinguishable compositions by amino acid analysis following acid hydrolysis, and were therefore assumed to represent the same peptide. Such doublets are common for products of synthesis by the Fmoc procedure (Smith et al., 1992). Amino acid analysis of the purified peptide following acid hydrolysis gave the composition $R_{2.0}P_{1.1}G_{2.0}V_{1.1}E_{2.0}K_{0.8}T_{1.1}Y_{1.9}$; and following enzymatic hydrolysis by carboxypeptidase Y, a composition of $Y_{1.9}T_{1.1}E_{2.0}K_{0.8}V_{1.0}W_{0.6}G_{0.4}P_{<0.1}R_{0.1}$ was obtained.

The synthetic peptide was next modified with 1.5 M pyridoxal phosphate, and the pyridoxamine was reduced with sodium cyanoborohydride. The product of this modification was purified on reverse-phase HPLC. Several peaks of absorbance at 229 nm for the peptide now coincided with several peaks of absorbance at 313 nm for pyridoxamine. Each of these peaks was also submitted to amino acid analysis following acid hydrolysis, and their several compositions were indistinguishable. Consequently, they were pooled as a group. After purification, an absorption spectrum was taken on a solution of this pool of peptides. In addition to the absorption maximum at 285 nm from the tryptophan and tyrosines, there was an absorption maximum at 328 nm. The absorption maximum of pyridoxamine phosphate is 327 nm.

Using the molar extinction coefficients 5690 M^{-1} and 1280 M^{-1} for tryptophan and tyrosine, respectively (Edelhoch, 1967), at 280 nm and 8800 M^{-1} for pyridoxamine phosphate at 327 nm (Dawson et al., 1969; Sober, 1973), the molar concentration of one tryptophan and two tyrosines and the molar concentration of pyridoxamine phosphate were calculated to be 65 μM and 66 μM, respectively. A portion of this solution was submitted to quantitative amino acid analysis following acid hydrolysis, and the concentration of the peptide was calculated to be 52 μM. The yield of lysine in this amino acid analysis was only 20% relative to those of the other amino acids in the peptide, and this result suggested that the lysine had been modified.

The *N*-acetylarginine was then removed by digestion with trypsin to produce the peptide RPGGWVEK(Hpyr)ETYY.

Because none of the peptide ETYY was observed at its characteristic relative mobility upon HPLC, it could be concluded that trypsin was unable to hydrolyze the peptide at the lysine because it was modified. This new peptide eluted as a multiplet at 28%–30% acetonitrile on C_{18} reverse-phase HPLC run in 0.05% TFA (Figure 1) and had a composition of $R_{1.2}P_{1.0}G_{2.0}V_{1.0}E_{2.1}K_{0.3}T_{0.8}Y_{1.8}$ as determined by amino acid analysis following acid hydrolysis. This peptide also coeluted on HPLC with multiple peaks of absorbance at 313 nm and had an optical absorption maximum at 329 nm of the same magnitude as that at 285 nm. The absorption spectrum, the coelution of absorption at 313 nm and 229 nm, and the fact that the peptide was not susceptible to tryptic digestion at the lysine demonstrated that the lysine in the peptide had been modified by the pyridoxal phosphate and the borohydride to produce the lysylpyridoxamine (λ_{max} = 327 nm). This peptide was bound by the immunoadsorbent specific for the carboxy-terminal amino acid sequence -ETYY (Bayer, 1990) and could be released from the immunoadsorbent at pH 2.5. This result indicates that its carboxy terminus was unmodified.

Location of Lysine 1012 Using 1.2% Cholate. Sealed, right-side-out vesicles were routinely obtained by floating microsomes from a homogenate of ovine renal medullas on a gradient of sodium ditrizoate (Forbush, 1982; Kyte et al., 1987). Addition of increasing amounts of cholate to these sealed vesicles, at the concentration (17 mg of protein mL^{-1}) at which the labeling would be performed, first caused an increase in enzymatic activity, which was presumably due to increased access to the active site, followed by a decrease in enzymatic activity, which was due to denaturation of the enzyme (Kyte, 1971). A concentration slightly less than the range of maximal activation, 1.2% cholate, [approximately 0.7 mg of cholate (mg of protein)$^{-1}$], was used in these experiments. At this concentration, the increase in enzymatic activity, referred to as the latency, was always at least 8-fold. This measurement indicates that in the absence of cholate the majority of the vesicles were sealed and that, upon addition of the detergent, the vesicles were rendered permeable to the ligands necessary for enzymatic function.

Sealed vesicles (29 mg of protein in each sample) were modified in the presence and absence of 1.2% cholate with 12 mM pyridoxal phosphate and 12.5 mCi of Na[^{3}H]BH_4 in each aliquot (1.7 mL). The modified α polypeptide was purified and digested with trypsin. The peptides were purified by passing each sample separately over the immunoadsorbent that could recognize the carboxy-terminal amino acid sequence -ETYY (Bayer, 1990) of the synthetic model peptide RPGGWVEK(Hpyr)ETYY. After the immunoadsorbent was washed with phosphate-buffered saline (>50 mL), the bound peptides were eluted with 1 M acetic acid and submitted to HPLC (Figure 1).

The upper chromatogram (Figure 1A) is that for the modified, synthetic peptide RPGGWVEK(Hpyr)ETYY with which the other samples were supplemented to act as a marker for the modified, carboxy-terminal peptide. The chromatogram of the peptides isolated from the sample to which no cholate had been added (Figure 1B) contained two peaks of interest. The first peak (peak a, Figure 1B) was determined by amino acid analysis following acid hydrolysis to have a composition corresponding to the peptide ETYY. It also had the relative mobility of synthetic ETYY. This peptide had been released from α polypeptides on which lysine 1012 had not been modified and which were, therefore, susceptible to digestion by trypsin at that lysine. This short peptide functioned as an internal standard in these experiments to account for any inequalities in the amount of α polypeptide present in the two samples and any differences in the yield of protein at each step in the purification, its digestion, and the purification of the peptides. The amount of this peptide in each run was quantified by amino acid analysis following acid

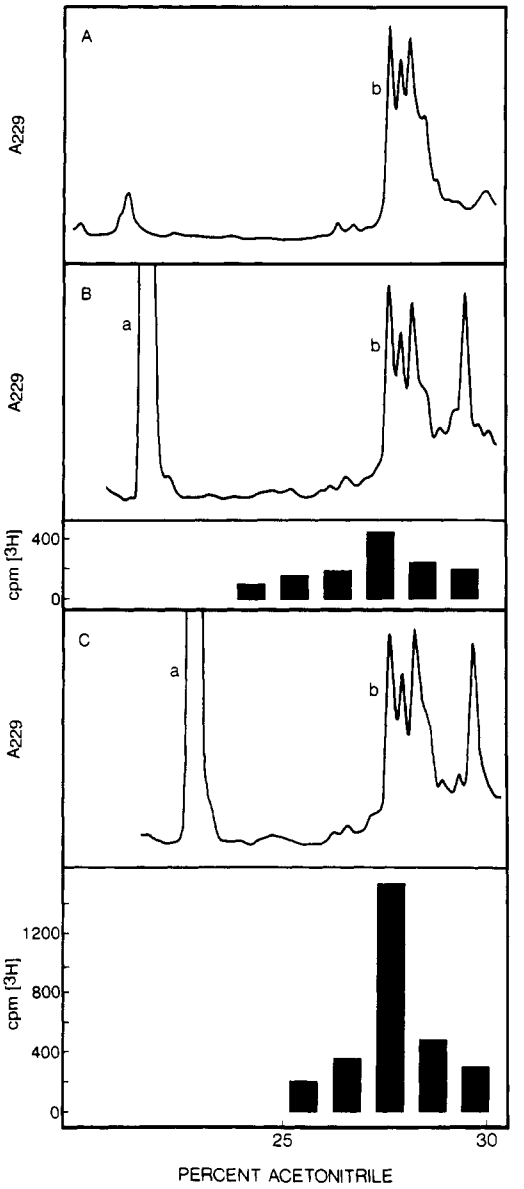

FIGURE 1: Incorporation of [^{3}H]pyridoxyl into lysine 1012. The respective samples of isolated, modified α polypeptide were digested with trypsin and then passed over an immunoadsorbent (21-nmol capacity) that recognized the carboxy-terminal sequence ETYY. Bound peptides were eluted with 1 M acetic acid, and the acid eluates were submitted to HPLC on a Vydac C_{18} reverse-phase column (0.46 cm × 25 cm). A linear gradient was developed by increasing the amount of acetonitrile (containing 0.017% TFA) at a rate of 1% min^{-1} from 0% to 40% acetonitrile at a flow rate of 1 mL min^{-1}. Absorbance was monitored continuously at 229 nm. The effluent from the HPLC was collected in 1-mL fractions, and the amount of tritium in fractions from 25% to 35% acetonitrile was determined by liquid scintillation (bar graphs). The effluent from 21% to 25% acetonitrile was pooled and submitted to amino acid analysis following acid hydrolysis to quantify the amount of ETYY. (A) Synthetic RPGGWVEK(Hpyr)ETYY submitted directly to HPLC. (B) Tryptic digest from sealed vesicles to which no cholate had been added. (C) Tryptic digest from sealed vesicles to which 1.2% cholate had been added prior to modification. Peak a in each chromatogram is the peak of absorbance due to ETYY; peak b in each chromatogram is the absorbance due to purposely added synthetic RPGGWVEK(Hpyr)ETYY, which designates the relative mobility of the cpm of RPGGWVEK([^{3}H]Hpyr)ETYY from the modified enzyme (bar graphs).

Table I: Specific Radioactivity of RPGGWVEK(^{3}Hpyr)ETYY[a]

		+detergent		−detergent		
expt	latency[b]	cpm	nmol of ETYY	cpm	nmol of ETYY	$(cpm^+)(nmol^-)/(cpm^-)(nmol^+)$[c]
		saponin experiments, sealed vesicles				
1	13	5640	8.9	1013	12	7.3
2	8	870	4.2	240	4.7	4.1
		cholate experiments, sealed vesicles				
3	8	1950	4.1	610	4.1	3.2
4	11	1770	6.8	460	6.7	3.8
5[d]	12	340	4.8	67	3.9	4.2
		cholate experiments, open vesicles				
6	1.5	150	0.12[e]	1130	1.1[e]	1.3
7	1.4	320	2.8[e]	420	2.8[e]	0.75

[a] The specific radioactivity for RPGGWVEK(^{3}Hpyr)ETYY was determined from experiments similar to those described in Figure 1. [b] Latency is the ratio of enzymatic activity of vesicles in samples to which detergent was added and those in which it was omitted. A high latency indicated that the majority of the vesicles were sealed. [c] This column contains ratios of the specific radioactivities in samples to which detergent had been added and omitted, respectively. [d] In this experiment, meglumine ditrizoate was used in place of sodium ditrizoate. [e] These numbers are given in terms of the area (cm^2) for the peaks of absorbance on the chromatograms due to difficulties in obtaining accurate values from amino acid analysis following acid hydrolysis.

hydrolysis. At the position of the second peak (peak b, Figure 1B), which is the peak of absorbance for the synthetic peptide, there was a small peak of radioactivity. It was assumed that the counts per minute in this designated peak of radioactivity divided by the nanomoles of ETYY on the chromatogram was an adequate measurement of the specific radioactivity of the lysine in the original, intact protein. This peak of radioactivity was 3.2-fold greater in specific radioactivity on the chromatogram of peptides from the sample to which cholate had been added prior to modification (Figure 1C). The specific radioactivities were 610 cpm $nmol^{-1}$ and 1950 cpm $nmol^{-1}$ in the absence and presence of cholate, respectively. In a series of several equivalent experiments the specific radioactivity increased 3-fold to 4-fold when cholate had been added to the sealed vesicles (Table I).

Identical experiments, using opened vesicles (with latencies of 1–2) in place of sealed vesicles, were then performed as a control (Dwyer, 1991). In this case, no difference in specific radioactivity between the vesicles with and without cholate was expected because the vesicles were already permeable to all the reagents, and none was observed (Table I). These results demonstrate that cholate did not effect the increase in specific radioactivity observed with the sealed vesicles by simply dissolving the protein, because there was no increase in the specific radioactivity of lysine 1012 when cholate was added to unsealed vesicles.

Similar experiments were also performed using saponin in place of cholate, as was done by Bayer (1990). Even larger increases in specific radioactivity were obtained when saponin was added to sealed vesicles (Table I). There was, however, an apparent increase in the specific radioactivity of the labeled, carboxy-terminal peptide (by more than 10-fold) when the saponin was added to open vesicles. This suggested that some of the increase in specific radioactivity seen with sealed vesicles could have been due to indirect effects of the saponin. There was also a loss of protein in samples to which saponin had been added, which also made the experiments equivocal. For these reasons, cholate was used rather than saponin for the majority of the experiments.

To provide additional evidence that the radioactivity in the peaks b in panels B and C of Figure 1 represented the labeled, carboxy-terminal peptide RPGGWVEK(^{3}Hpyr)ETYY from the α polypeptide, the experiment was repeated with the following modification. The regions of the chromatogram surrounding the synthetic peptide (peak b, Figure 1) were pooled and digested with thermolysin. Digestion with thermolysin should have produced the peptides RPGGW and VEK(^{3}Hpyr)ETYY. The movement of the peak of radioactivity from the position of the longer peptide to the position of the shorter peptide, VEK(^{3}Hpyr)ETYY, would indicate that the radioactivity was covalently attached to the lysine in RPGGWVEKETYY.

The synthetic peptide RPGGWVEK(Hpyr)ETYY was digested alone with thermolysin to determine the relative mobilities of its two fragments (Figure 2A). The peak of absorbance at 23% acetonitrile (peak a) had an amino acid composition corresponding to that of the peptide VEKETYY; the peak of absorbance at 25% acetonitrile (peak b) had an amino acid composition corresponding to that of the peptide RPGG. The third peak (peak c) was the undigested, long peptide.

The chromatograms of the thermolytic digests (Figure 2B,C) of the pooled material (corresponding to peak b, Figure 1B,C) each displayed a new peak of radioactivity at the position of the peptide VEK(Hpyr)ETYY. When cholate was added prior to modification of the protein, at least a 3.8-fold increase in the specific radioactivity of both the undigested peptide (peak c, Figure 2B,C) and the shorter peptide (peak a, Figure 2B,C) was observed (Table II).

These results are consistent with the conclusion that the peaks of radioactivity coeluting with the synthetic peptide RPGGWVEK(Hpyr)ETYY represent the modified peptide RPGGWVEK(^{3}Hpyr)ETYY, from the modified enzyme. That the radioactivity was bound by the immunoadsorbent and that it coeluted with the synthetic peptide on the first chromatogram are also consistent with this conclusion. Because the specific radioactivity increased when sealed vesicles were treated with cholate but not when open vesicles were treated with cholate (Table I), these results demonstrate that the carboxy terminus of the α polypeptide of $(Na^+ + K^+)$-ATPase is located on its cytoplasmic surface.

Examination of the Location of Lysine 905. The foregoing results were contrary to those that had been published earlier using similar techniques (Bayer, 1990), so it was important also to reexamine the earlier experimental results relevant to the location of lysine 905. This amino acid is contained in the peptide QRKIVE which is released when the α polypeptide is digested with the proteinase from *S. aureus* strain V8.

Sealed vesicles (31 mg of protein in 1.8 mL for each situation) were labeled with pyridoxal phosphate and Na-[^{3}H]BH_4 in the presence and absence of saponin, as had been done previously (Bayer, 1990) with concentrations of 12 mM pyridoxal phosphate (previously 6 mM) and 0.4% saponin (previously 0.2%). The α polypeptide was isolated and digested with the proteinase from *S. aureus* strain V8. The glutamine was cyclized by adding sodium phosphate at pH 8 to 0.25 M and incubating at 37 °C for 20–24 h (Gilbert, 1949). Each cyclized digest was then separately passed over an immunoadsorbent that recognized the sequence pyroERXIVE (Bayer, 1990), where X indicates the point of attachment of the hapten. The peptides retained by the immunoadsorbent were eluted with 1 M acetic acid. When these eluates were submitted to HPLC, two peaks of significance were observed (Figure 3). The second (peak b) displayed the amino-terminal, amino acid sequence RYA (first cycle R, 9 pmol; G, 12 pmol; S, 5 pmol; A, 4 pmol; second cycle Y, 6 pmol; R, 4 pmol; G,

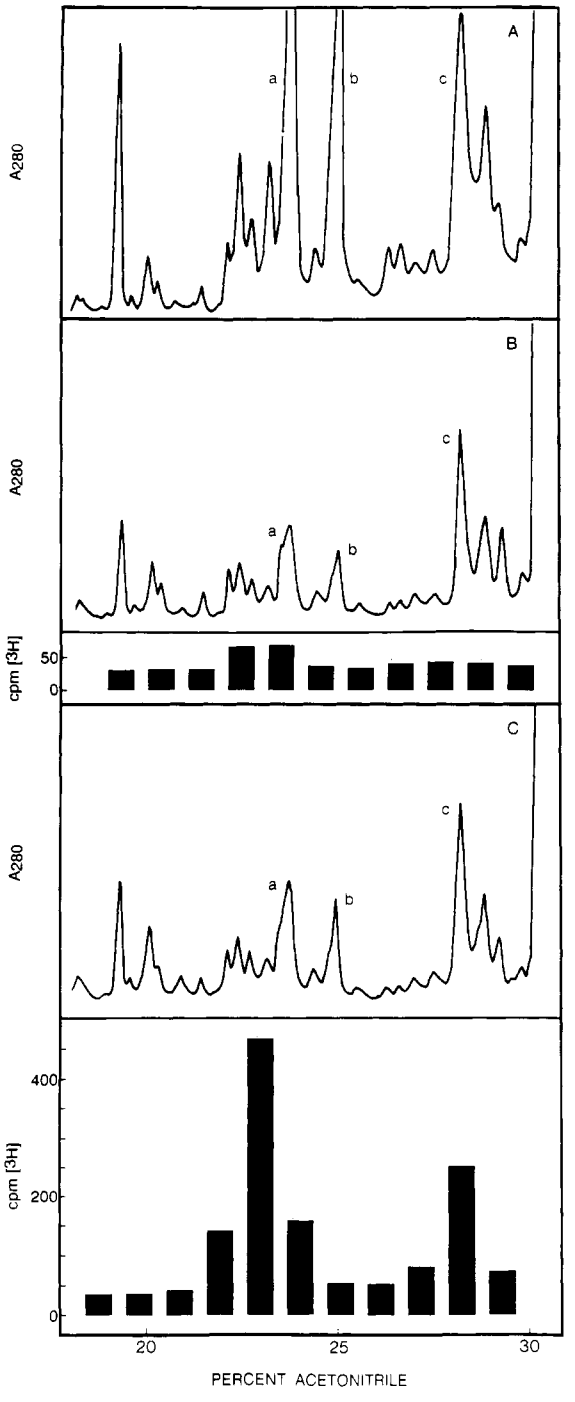

FIGURE 2: Digestion of RPGGWVEK([3]Hpyr)ETYY with thermolysin. Effluents from 27% to 30% acetonitrile from chromatograms identical to those presented in Figure 1 were pooled, digested with thermolysin, and resubmitted to reverse-phase HPLC under the same conditions as those described in Figure 1. Absorbance was monitored continuously at 280 nm. The effluent was collected in 1-mL fractions, and the amount of tritium in fractions from 10% to 31% acetonitrile was determined by liquid scintillation (bar graphs). (A) Synthetic RPGGWVEK(Hpyr)ETYY as in panel A of Figure 1, digested with thermolysin. (B) Pool from tryptic digest of sealed vesicles to which no cholate was added prior to modification, as in panel B of Figure 1, digested with thermolysin. (C) Pool from tryptic digest of sealed vesicles to which 1.2% cholate was added prior to modification, as in panel C of Figure 1, digested with thermolysin. Peak a is the peak of absorbance due to VEK(Hpyr)ETYY, peak b is the peak of absorbance due to RPGG, and peak c is the absorbance due to RPGGWVEK(Hpyr)ETYY. Each of these peaks of absorbance derive from the synthetic peptide included in the initial samples as in Figure 1, and they were identified in separate experiments by their amino acid compositions.

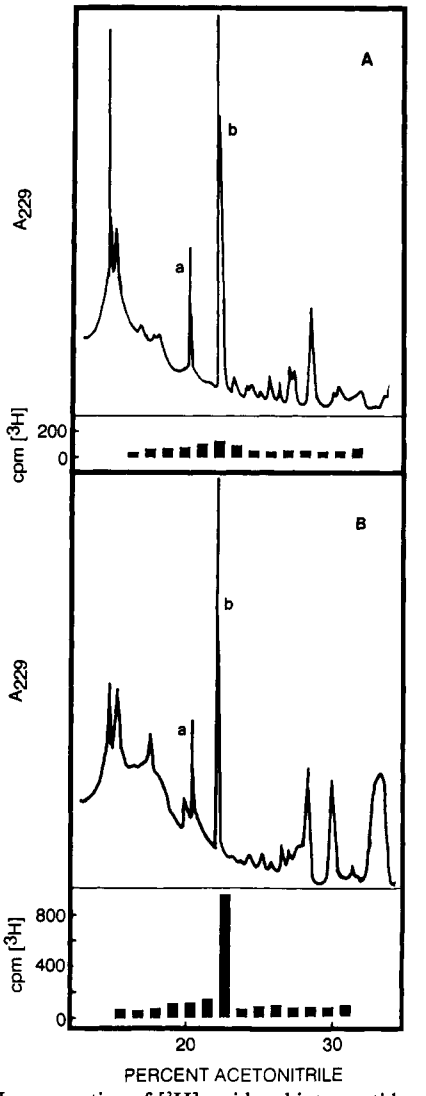

FIGURE 3: Incorporation of [^{3}H]pyridoxyl into peptides recognized by the immunoadsorbent specific for the amino acid sequence pyroERXIVE. The respective samples of isolated, modified α polypeptide were digested with the proteinase from *S. aureus* strain V8, the glutamines were cyclized, and the samples were passed over an immunoadsorbent which recognized the sequence pyroERXIVE. The bound peptides were eluted with 1 M acetic acid, and the acid eluates were submitted to HPLC on a Vydac C_{18} reverse-phase column (0.46 cm × 25 cm). A linear gradient was developed by increasing the amount of acetonitrile (containing 0.017% TFA) at a rate of 1% min^{-1} from 0% to 35% acetonitrile at a flow rate of 1 mL min^{-1}. Absorbance was monitored continuously at 229 nm. The effluent from the HPLC was collected in 1-mL fractions, and the amount of tritium in 1-mL fractions from 16% to 35% acetonitrile was determined by liquid scintillation (bar graphs). (A) *S. aureus* strain V8 proteinase digest from sealed vesicles to which no saponin had been added. (B) *S. aureus* strain V8 proteinase digest from sealed vesicles to which saponin had been added prior to modification with pyridoxal phosphate. Peak a in each chromatogram is the peak of absorbance due to pyroERKIVE; peak b in each chromatogram is the peak of absorbance due to RYAKIVE.

4 pmol; third cycle A, 3 pmol) by automated Edman degradation. Examination of the amino acid sequence of the α polypeptide indicated that this corresponded to the peptide RYAKIVE, which is also released from the α polypeptide by digestion with the proteinase from *S. aureus* strain V8. The first peak of absorbance (peak a) had the same mobility on the HPLC as synthetic pyroERKIVE; it displayed very low yields of multiple signals at each step when sequenced and this was consistent with the amino terminus being blocked by pyroglutamate. A separate sample of the same peptide

Table II: Specific Radioactivity of VEK(^{3}Hpyr)ETYY and RPGGWVEK(^{3}Hpyr)ETYY[a]

		+cholate			−cholate				
		cpm			cpm			+cholate/−cholate[e]	
expt	latency[b]	V[c]	R[d]	nmol of ETYY	V	R	nmol of ETYY	V	R
1	21	280	65	7	87	20	6	3.8	3.9
2[f]	10	660	300	4.7	84	36	5.2	7.1	7.6

[a] The specific radioactivities of VEK(^{3}Hpyr)ETYY and RPGGWVEK(^{3}Hpyr)ETYY were determined from experiments similar to those described in Figure 2. [b] Latency is the ratio of enzymatic activity of vesicles in samples to which detergent was added and those in which it was omitted. A high latency indicated that the majority of the vesicles were sealed. [c] This column contains values for VEK(^{3}Hpyr)ETYY. [d] This column contains values for RPGGWVEK(^{3}Hpyr)ETYY. [e] This column contains the ratios of specific radioactivities of samples to which detergent had been added and omitted, respectively. The following formula was used to correct for any losses or inequalities of protein or peptide during the experiment: $[(cpm^+)(cpm^-)^{-1}][(\text{nmol of ETYY}^-)(\text{nmol of ETYY}^+)^{-1}][(\text{height of peak b, Figure 1C}^+)(\text{height of peak b, Figure 1B}^-)^{-1}][(\text{area of peak a, Figure 2B}^-)(\text{area of peak a, Figure 2C}^+)^{-1}]$. [f] In this experiment, meglumine ditrizoate was used in place of sodium ditrizoate.

obtained from a digest of the α polypeptide had the amino acid composition $E_{1.9}R_{1.5}K_{1.1}I_{0.6}V_{0.7}$ following acid hydrolysis, as expected from pyroERKIVE.

The radioactivity associated with the peak of absorbance of the peptide RYAKIVE increased up to 15-fold when saponin was added (Figure 3). The specific radioactivity was 2700 cpm cm^{-2} in the presence of saponin and 175 cpm cm^{-2} in its absence where cm^2 are the units of area of the peaks of absorbance. Unfortunately, there was little radioactivity associated with the peak of absorbance from the peptide pyroERKIVE. Because the radioactivity associated with the peptide RYAKIVE overlapped that associated with the peptide pyroERKIVE, it was uncertain if this radioactivity was specific to pyroERKIVE.

These results demonstrate that the previous assignment of lysine 905 to the cytoplasmic surface (Bayer, 1990) was mistaken because of the presence of RYAK(^{3}Hpyr)IVE in the eluates from the immunoadsorbents. At present, because it has been difficult to modify lysine 905, its position with respect to the bilayer is uncertain.

DISCUSSION

In a report from our laboratory, Bayer (1990) recently concluded that the position of the carboxy terminus of $(Na^+ + K^+)$-ATPase is extracytoplasmic. This conclusion was based on a procedure identical in part to that used in the present experiments. As was done here, a small percentage of the lysines of the enzyme were modified with pyridoxal phosphate and Na[^{3}H]BH_4 while it was in its native conformation, the modified enzyme was digested with trypsin to yield the same specific, modified peptides, and those peptides were isolated with the same immunoadsorbent used in the present studies. The conclusion drawn from these earlier experiments has since been contradicted in studies of other members of the superfamily of transporting ATPases. A cytoplasmic location for the carboxy terminus has been reported for calmodulin-activated, plasma membrane Ca^{2+}-transporting ATPase (James et al., 1988), endoplasmic reticular Ca^{2+}-transporting ATPase (Matthews et al., 1989), and plasma membrane H^+-transporting ATPase (Hennessey & Scarborough, 1990; Mandala & Slayman, 1989). Because the members of this superfamily share considerable sequence homology, they must also share very similar structures (Chothia & Lesk, 1986). It was therefore considered necessary to reexamine the results of Bayer (1990).

The method developed by Kyte et al. (1987) and used in Bayer (1990) has been improved to permit a more rigorous analysis of the peptide of interest in the last steps of the experiment. The first improvement, described by Dwyer (1991), is to submit the acid eluates from the immunoadsorbents to HPLC. This has made it possible both to quantify the unmodified peptide that was bound and released by the immunoadsorbent and to verify that the radioactivity bound and released by the immunoadsorbent has the mobility expected for the modified peptide. In the experiments described by Dwyer (1991), the peptides of interest were from digestions that produced modified and unmodified peptides of the same relative mobility on HPLC so that the coincidence of the peak of radioactivity and the peak of absorbance verified that the peak of radioactivity represented the modified peptide. In the present experiments, because trypsin was used to produce the modified peptide, which was alkylated at lysine, the modified peptide had a dramatically different mobility on HPLC. This led to the second improvement, which was the synthesis of an appropriate standard for the chromatography. The entire peptide that would be released upon digestion of the modified α polypeptide was synthesized and modified with pyridoxal phosphate to use as a standard. As the parent sequence was -IIRRRPGGWVEKETYY, digestion with trypsin should have given two products recognized by the immunosorbent, ETYY and RPGGWVEK(^{3}Hpyr)ETYY, the former from unmodified and the latter from modified α polypeptide. Because the majority (about 95%) of the α polypeptide was unmodified (Kyte et al., 1987) at the concentration (12 mM) of pyridoxal phosphate used, the only product that could be chemically identified was the peptide ETYY (peak a in Figure 1). The synthesis of the peptide RPGGWVEK(Hpyr)ETYY as a standard made it possible to identify the peak of radioactivity corresponding to the small amount of the modified peptide from the modified α polypeptide by its relative mobility on HPLC.

In this regard, it is of interest that the peak of radioactivity representing the modified peptide from $(Na^+ + K^+)$-ATPase coincided with the first of the several components present in the synthetic, modified peptide (Figure 1). It has been demonstrated previously that the most common side products of Fmoc synthesis are butylated peptides (Smith et al., 1992). Such side products should have a slower mobility on HPLC than that of the unbutylated parent peptide, which should be the first component to elute. It is this first component of the synthetic mixture that has a relative mobility coinciding with the radioactivity that has been assigned to the peptide RPGGWVEK(^{3}Hpyr)ETYY, which should be unbutylated because it was derived from the enzyme itself. When the modified peptide represented by this peak of radioactivity was submitted to thermolytic digestion, the radioactivity moved to the elution position of the synthetic peptide VEK(Hpyr)-ETYY, as was expected for digestion of the correct peptide. This result confirms that the modified peptide represented by the peak of radioactivity on the elution profile in Figure 1 is RPGGWVEK(^{3}Hpyr)ETYY.

Quantification of the radioactivity in RPGGWVEK-(^{3}Hpyr)ETYY and the amount of ETYY on HPLC permitted a determination of specific radioactivity, which corrects for any relative losses of protein or peptide during the experiment. Dwyer (1991) based this calculation of specific radioactivity on the counts per minute of the peak of radioactivity divided by the height of the corresponding peak of absorbance coeluting with it. In those experiments, the products of digestion of the modified and unmodified protein had the same mobility on HPLC. The peak of absorbance, representing the unmodified peptide, identified the position of the modified peptide. The height of the peak of absorbance was taken to represent the amount of the specific peptide present at the final step of the procedure. In the present experiments, the unmodified α polypeptide gave a product, ETYY, that had a mobility on HPLC different from that of the modified peptide. This made it possible to collect separately and quantify the peak of absorbance from the peptide, ETYY, using acid hydrolysis followed by amino acid analysis, which should be more accurate than simply the area of absorbance. It was assumed that the molar amount of ETYY present represented the yield of tryptic peptides through the last step of the experiment.

When cholate was added to samples prior to modification, the specific radioactivity of lysine 1012 increased at least 3-fold (Table I). This indicated that the cholate permitted increased access of the labeling reagents to this amino acid residue and that lysine 1012 must therefore be on the cytoplasmic side of (Na^++K^+)-ATPase. Consistent with this conclusion was the observation that the specific radioactivity of the peptide VEK-(^{3}Hpyr)ETYY from the modified protein also increased at least 3.8-fold (Table II) when cholate was added prior to modification.

Because the conclusions reached about the position of lysine 1012 disagreed with those of Bayer (1990), the position of lysine 905 which is contained in the peptide QRKIVE was also reexamined. In the present experiments, it was discovered that the immunoadsorbent against pyroERXIVE, which also was the same one used by Bayer, also recognized the peptide RYAK(^{3}Hpyr)IVE, a peptide released by digestion of the modified α polypeptide from the region of the amino acid sequence of the α polypeptide between glutamate 465 and glutamate 472. These two peptides, pyroERK(^{3}Hpyr)IVE and RYAK(^{3}Hpyr)IVE, had very similar mobilities on HPLC; and, while a large amount of the [^{3}H]pyridoxamino group could be incorporated into RYAKIVE, very little was incorporated into the peptide of interest, pyroERKIVE. Although it can be concluded that lysine 469 is on the cytoplasmic surface of the α subunit, a disposition that is not surprising, the position of lysine 905 is uncertain at this time. Attempts to modify it with other impermeant reagents have so far been unsuccessful.

Two groups have recently reported that a region of the endoplasmic reticular Ca^{2+}-ATPase, corresponding to the area surrounding lysine 905, is located on the extracytoplasmic surface of the enzyme (Clarke et al., 1990; Matthews et al., 1990). In these experiments, an immunoglobulin directed against this region was found to bind to the protein in the presence of a detergent but could not bind to intact vesicles of sarcoplasmic reticulum. This could indicate that this region of the amino acid sequence is located on the extracycloplasmic surface of all the enzymes of this superfamily. There are, however, a number of uncertainties concerning this technique of epitope mapping. In our experience, this region is very inaccessible, even to small impermeant reagents, let alone immunoglobulins, in the native structure of the enzyme. It is possible that the immunoglobulins used in these other experiments may have preferentially recognized a small, unrepresentative population of denatured protein present in the samples. The possibility has been raised that in such experiments a sequence may be sterically inaccessible to the immunoglobulin or folded in a manner which is not recognized by the immunoglobulins until surfactants are added (McCrea et al., 1987). Additionally, in studies on the nicotinic acetylcholine receptor, there have been reports of cross-reactivity of some antipeptide immunoglobulins with other regions of the protein (Maelicke et al., 1989).

Therefore, it is necessary to examine the position of lysine 905 using an alternative technique. The experiments presented here avoid some of the problems of epitope mapping because the peptide recognized by the immunoadsorbent can be purified and characterized on HPLC. It should be emphasized that the immunoadsorbents used in the present procedures are not used to recognize epitopes in the native protein, only to isolate modified peptides from enzyme labeled with small impermeant reagents while in its native conformation.

ACKNOWLEDGMENT

I am grateful to Dr. Jack Kyte, in whose laboratory this research was performed, for his advice, support, and guidance. It also thank Siv Garrod and Dr. Susan Taylor for performing the peptide sequencing.

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