or initiation at the capped 5′ end relative to that at the internal site.

Additional experiments will be required to elucidate the potential role of CBP and other initiation factors in the internal initiation of translation. Preliminary in vitro experiments suggest that internal initiation may be somewhat less sensitive to the polio virus induced cleavage of the CBP complex when assayed in extracts of polio-infected HeLa cells (R. Herman and R. Lundquist, unpublished observations). The function of the 7K protein, which may be a minor component of the VSV virion (unpublished observations), also remains to be determined.

ACKNOWLEDGMENTS

I gratefully acknowledge the dedicated technical assistance provided by C. Rossi. I also thank L. A. Ball, S. U. Emerson, M. Hunt, J. A. Lesnaw, R. Lundquist, S. A. Moyer, J. Rose, J. Shaffer, N. Sonenberg, and M. Williams for providing reagents, invaluable advice, and encouragement during the course of this work.

Registry No. 7mGpppG, 110971-03-4; K, 7440-09-7; Mg, 7439-95-4.

REFERENCES

Edery, L., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H., & Sonenberg, S. (1983) *J. Biol. Chem. 258*, 11398–11403.

Edery, I., Lee, K. A. W., & Sonenberg, N. (1984) *Biochemistry 23*, 2456–2462.

Gallione, C. J., Greene, J. R., Iverson, L., & Rose, J. K. (1981) *J. Virol. 39*, 529–535.

Hassin, D., Korn, R., & Horwitz, M. S. (1986) *Virology 155*, 214–224.

Herman, R. C. (1986) *J. Virol. 58*, 797–804.

Kozak, M. (1983) *Microbiol. Rev. 47*, 1–45.

Kozak, M., & Shatkin, A. J. (1977) *J. Biol. Chem. 252*, 6895–6908.

Laemmli, U. K. (1970) *Nature (London) 222*, 680–685.

Lodish, H. F., & Rose, J. K. (1977) *J. Biol. Chem. 252*, 1181–1188.

Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.

Nagy, E., Duncan, R., Krell, P., & Dobos, P. (1987) *Virology 158*, 211–217.

Shih, D. S., Park, I.-W., Evans, C. L., Jaynes, J. M., & Palmenberg, A. C. (1987) *J. Virol. 61*, 2033–2037.

Sonenberg, N. (1981) *Nucleic Acids Res. 9*, 1643–1656.

Sonenberg, N., Guertin, D., Cleveland, D., & Trachsel, H. (1981) *Cell (Cambridge, Mass.) 27*, 563–572.

Sonenberg, N., Edery, I., Darveau, A., Humbelin, M., Trachsel, H., Hershey, J. W. B., & Lee, K. A. W. (1983) in *Protein Synthesis* (Abraham, A. K., Eikham, T. S., & Pryme, I. F., Eds.) pp 23–43, Humana, Clifton, NJ.

Weber, L. A., Hickey, E. D., & Baglioni, C. (1978) *J. Biol. Chem. 253*, 178–183.

Demonstration That Lysine-501 of the α Polypeptide of Native Sodium and Potassium Ion Activated Adenosinetriphosphatase Is Located on Its Cytoplasmic Surface†

Jack Kyte,* Kai-yuan Xu, and Robert Bayer

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

Received June 3, 1987; Revised Manuscript Received August 18, 1987

ABSTRACT: Evidence that the peptide HLLVMKGAPER, which can be released from intact sodium and potassium ion activated adenosinetriphosphatase by tryptic digestion, is located on the cytoplasmic surface of the native enzyme has been obtained. An immunoadsorbent directed against the carboxy-terminal sequence of this tryptic peptide has been constructed. The peptide KGAPER was synthesized by solid-phase techniques. Antibodies against the sequence -GAPER were purified by immunoadsorption, using the synthetic peptide attached to agarose beads. These antibodies, in turn, were coupled to agarose beads to produce an immunoadsorbent. Sealed, right-side-out vesicles, prepared from canine kidneys, were labeled with pyridoxal phosphate and sodium [^{3}H]borohydride in the absence or presence of saponin, respectively. A tryptic digest of these labeled vesicles was passed over the immunoadsorbent. Large increases in the incorporation of radioactivity into the peptides bound by the immunoadsorbent were observed in the digests obtained from the vesicles exposed to saponin. From the results of several control experiments examining the labeling reaction as applied to these vesicles, it could be concluded that this increase in incorporation resulted only from the access that the reagents gained to the inside of the vesicles in the presence of saponin and that the increase in the extent of modification was due to the cytoplasmic disposition of this segment in the native enzyme.

The general problem of identifying membrane-spanning sequences in membrane-spanning proteins is supposed to have both a computational solution and an experimental solution. Although several other methods have been proposed, which differ in detail (Argos et al., 1982; Guy, 1984; Kuhn & Leigh, 1985; Eisenberg et al., 1984; Engelman et al., 1986), our own algorithm (Kyte & Doolittle, 1982) is illustrative of the computational solution. Each amino acid in a given segment 19 residues in length is assigned a numerical value reflecting its

† This research was supported by Grant DMB-8413772 from the National Science Foundation, Grants GM-33962 and AM-07233 from the National Institutes of Health, and Grant-in-Aid AHA-81-1003 from the American Heart Association. R.B. was also supported by Postdoctoral Training Grant AM-07233 from the National Institutes of Health.

0006-2960/87/0426-8350$01.50/0 © 1987 American Chemical Society

hydropathy. When the mean value of these assignments is above a certain threshold, there is a high probability that the segment spans the membrane in the native structure of the protein. This criterion was established with membrane-spanning sequences from bacteriorhodopsin, glycophorin, and the coat protein of M13 phage, and it was successful in predicting (Williams et al., 1984), before they were ascertained (Deisenhofer et al., 1985), the membrane-spanning sequences of the photosynthetic reaction center. The experimental solution to the problem of identifying membrane-spanning sequences is to modify the protein of interest with a hydrophobic reagent that partitions preferentially into the bilayer and to isolate the peptides into which it has inserted (Bercovici & Gitler, 1978; Bayley & Knowles, 1980; Ross et al., 1982; Malatesta et al., 1983).

Both of these approaches have been applied to the α polypeptide of sodium and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase],[1] the membrane-spanning enzyme responsible for the coupled active transport of sodium and potassium (Kyte, 1981). Several complete sequences of this protein are now available from different species (Shull et al., 1985, 1986; Kawakami et al., 1985; Herrera et al., 1987), and two of the computerized algorithms (Kyte & Doolittle, 1982; Engelman et al., 1986) have been applied to them. Six, seven, or eight membrane-spanning segments have been proposed for this protein on the basis of such analyses. We have isolated five radioactive tryptic peptides of apparent length 70, 50, 35, 35, and 27 residues and with apparent amino-terminal sequence IATL-, VLGF-, LIXLA-, LFLF-, and MYLP-, respectively, from the α polypeptide modified in native canine, renal $(Na^+ + K^+)$-ATPase with the hydrophobic reagent [1-^{3}H]spiroadamantane 4,3′-diazirine (Nicholas, 1984). Although the amino-terminal sequences determined for two of these peptides appear to be incorrect, LFLF- instead of ILIF- and MYLP- instead of MYPL-, from the apparent lengths, the amino-terminal sequences, and the amino acid compositions (Nicholas, 1984), these five peptides can be assigned to residues 263–342, 545–589, 842–880, 946–972, and 973–998 in the sequence of the α polypeptide from renal $(Na^+ + K^+)$-ATPase (Shull et al., 1985). Each of these sequences contains 1 or 2 hydrophobic segments 20 residues in length. Two of these segments, those within residues 545–589 and 973–998, were picked out by neither of the algorithms in their several applications, and four of the segments picked out by one or both of the algorithms were not isolated in modified peptides. Because the computational solutions and the experimental solution are not in agreement among themselves, the identities of the membrane-spanning sequences of the α polypeptide of $(Na^+ + K^+)$-ATPase have yet to be established.

An alternative approach of making the decision of which segments of the sequence of a membrane-spanning protein cross the bilayer is to determine the disposition of selected regions of the sequence of the protein located outside the bilayer. If one region of the sequence is shown to be on the cytoplasmic surface of the native protein and an adjacent region is shown to be on the extracytoplasmic surface, then a membrane-spanning segment must be located between them. The immunochemical technique of raising antibodies directed against specific hydrophilic sequences from a membrane-spanning protein and using these antibodies to elucidate directly its topology has been exhaustively applied in at least one instance (Ratnam et al., 1986), but the results obtained have led to conclusions that seem sterically difficult to accept, a problem that raises doubts about this approach. Proteolytic digestion of the native form of a membrane-spanning protein in sealed, oriented vesicles or intact cells often provides information about its topology (Jørgensen & Petersen, 1985), but such results are serendipitous. For instance, no proteolytic cleavage of the α polypeptide on the extracytoplasmic surface of native $(Na^+ + K^+)$-ATPase has been identified. Our approach to a systematic determination of the topology of the α polypeptide in native $(Na^+ + K^+)$-ATPase combines the chemical labeling of sealed, right-side-out vesicles with impermeant reagents and the immunochemical identification of the products of the labeling reaction. The method has been applied to determine the location of Lys_{501} in the α polypeptide of $(Na^+ + K^+)$-ATPase, a residue not far from one of the tryptic peptides (545–589) labeled with spiroadamantane 4,3′-diazirine (Nicholas, 1984).

It has been shown that fluorescein 5′-isothiocyanate is capable of inactivating $(Na^+ + K^+)$-ATPase (Karlish, 1980), at least in part due to the modification of Lys_{501}. Lysine-501 can be released in its modified form within the peptide HLLVMK(FITC)GAPER when the native enzyme, modified by fluorescein 5′-isothiocyanate, is digested with trypsin (Farley et al., 1984), and this feature has simplified the current experiments. The results presented here provide direct evidence that Lys_{501} is located on the cytoplasmic surface of $(Na^+ + K^+)$-ATPase.

Experimental Procedures

Materials. Pyridoxal phosphate, saponin, adenosine 5′-triphosphate, histidine, soybean trypsin inhibitor, carboxypeptidase B, deoxycholate, thermolysin, $NaBH_4$, and 2-mercaptoethanol were purchased from Sigma Chemical Corp.; Sepharose 4B, chloromethylated polystyrene (Bio-Beads S-X1, 200–400 mesh, 1.37 mequiv g^{-1}), and Aminex A-5 were from Bio-Rad Corp.; derivatives of amino acids protected at their α-amino nitrogens with *tert*-butoxycarbonyl (Boc) groups were from Bachem Corp.; dicyclohexylcarbodiimide was from Aldrich Chemical Co.; 1-deoxy-1-(methylammonium)-D-glucitol 3,4-(diacetamido)-2,4,6-triiodobenzoate (Hypaque meglumine as a 60% solution) was from Winthrop–Breon Laboratories; trypsin that had been treated with *N*-(*p*-toluenesulfonyl)-L-phenylalanyl chloromethyl ketone was from Worthington Corp.; ninhydrin was from Pierce Chemical Corp.; bovine serine albumin was from Miles Diagnostics Corp.; acetonitrile (high-pressure liquid chromatographic grade) was from Fisher Chemical Corp.; glutaraldehyde (20% solution) was from ICN Corp.; Freund's adjuvant was from Difco Corp.; ammonium sulfate (enzyme grade) was from Schwarz/Mann Corp.; cyanogen bromide was from MCB Corp.; Sephadex G-50M was from Pharmacia Corp. These compounds were used directly. Sodium dodecyl sulfate ($NaDodSO_4$) was purchased from Calbiochem Corp. and recrystallized from 95% ethanol (Burgess, 1969); imidazole was recrystallized from benzene and then acetone; trifluoroacetic acid was redistilled after adding a small amount of H_2O; formic acid was redistilled; triethylamine and pyridine were redistilled after refluxing with phthalic anhydride (added as solid to 5%); phenyl isothiocyanate was redistilled under vacuum. Anhydrous hydrogen fluoride was condensed from the gas (Stewart & Young, 1969). Bovine serum albumin was reduced and carboxamidomethylated with iodoacetamide according to Nicholas (1984). Anthroylouabain (Fortes, 1977)

[1] Abbreviations: $(Na^+ + K^+)$-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); Boc, *tert*-butoxycarbonyl; $NaDodSO_4$, sodium dodecyl sulfate; [^{3}H]$NaBH_4$, sodium [^{3}H]borohydride; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetate.

was the gift of Dr. George Fortes, University of California at San Diego. Sodium [^{3}H]borohydride ([^{3}H]$NaBH_4$) was purchased as the solid from either New England Nuclear Corp. or Amersham Corp. A solution at 50–100 mM in 10 mM NaOH was prepared and either used immediately or stored at −70 °C until use. We have found that the [^{3}H]$NaBH_4$ deteriorates significantly under these storage conditions.

Preparation of (Na^+ + K^+)-ATPase and Sealed, Right-Side-Out Vesicles. Purified (Na^+ + K^+)-ATPase in open fragments of membrane was prepared by the method of Jørgensen (1974) with the modifications described by Winslow (1981). Sealed, right-side-out vesicles, containing (Na^+ + K^+)-ATPase, were prepared from canine renal microsomes (Kyte, 1971), whose specific enzymatic activity was 50 μmol of P_i $(mg \cdot h)^{-1}$, by flotation on solutions of 1-deoxy-1-(methylammonium)-D-glucitol 3,5-(diacetamido)-2,4,6-triiodobenzoate according to Forbush (1982). Either the gradient originally described by Forbush or a simple 16% solution of the 1-deoxy-1-(methylammonium)-D-glucitol 3,5-(diacetamido)-2,4,6-triiodobenzoate was used. The latter procedure seemed to yield preparations with a higher percentage of sealed vesicles.

Determination of (Na^+ + K^+)-ATPase Activity. Sealed vesicles were premixed for 12 min at room temperature with either saponin or deoxycholate added as concentrated solutions. Routinely, saponin was used at both 0.2% and 0.5% final concentrations, each of which would give the same activation. Portions were then removed and assayed for strophanthidin-sensitive (Na^+ + K^+)-ATPase activity (Kyte, 1971). Purified (Na^+ + K^+)-ATPase was assayed without preincubation.

Protein Concentration, Amino Acid Analysis, and Electrophoresis. Protein concentration was routinely performed by the method of Lowry et al. (1951) as modified by Kyte (1971) or by quantitative amino acid analysis as described by Moczydlowski and Fortes (1981). The concentration of reduced and carboxamidomethylated bovine serum albumin was determined from A_{280}. Samples of synthetic peptide were prepared for amino acid analysis by hydrolysis in 6 M HCl for 24 h at 110 °C or for 30 min at 155 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Shapiro et al., 1967) was performed by the modifications of Weber and Osborn (1969). Samples were prepared by suspending membranes in a dilute buffer, adding 2-mercaptoethanol to 0.1%, adding at least 5 μg of $NaDodSO_4$ (μg of protein)$^{-1}$, and exposing the sample either to 100 °C for 1 min or, if necessary, to room temperature for several hours. For scintillation counting, slices of polyacrylamide gels were prepared by the procedure of Drickamer (1976) as described by Munson (1983).

Proteolytic Digestions. Modified α polypeptide of (Na^+ + K^+)-ATPase was isolated from labeled vesicles by gel filtration (Kyte, 1972). The dodecyl sulfate was stripped from the polypeptide (Sharkey, 1983), and thermolysin (1:30 by weight) was added to the stripped polypeptide that had been dialyzed into 50 mM ammonium bicarbonate buffer, pH 8.0. After 6 h at 37 °C, an additional aliquot of thermolysin was added, and digestion was continued overnight.

Sealed, right-side-out vesicles labeled with [^{3}H]pyridoxamine phosphate were collected by ultracentrifugation, resuspended in 0.5% saponin, 1 mM ethylenediaminetetraacetate (EDTA), and 25 mM imidazolium chloride, pH 7.5, and digested (Farley et al., 1984) with trypsin (1:10 by weight). After 3 h, soybean trypsin inhibitor was added at a 2-fold weight excess over trypsin to stop the reaction, and the mixture was centrifuged at 45 000 rpm in a Beckman Ti50.2 rotor for 1 h. The supernatant was centrifuged again at 45 000 rpm for 30 min and lyophilized.

Synthetic peptide, immobilized on agarose beads, was submitted to digestion by carboxypeptidase B. Settled beads (100 μL) were mixed with a solution of carboxypeptidase B so that the final concentrations would be 40 μg mL^{-1} carboxypeptidase B and 0.2 M *N*-ethylmorpholinium acetate, pH 8.5. The sample was incubated at 37 °C for 1 h, the beads were removed, the pH was lowered to 2.2, and the sample was submitted directly to amino acid analysis.

Cation-Exchange Chromatography and High-Pressure Liquid Chromatography (HPLC). Synthetic peptide or proteolytic digests were dissolved in 20–60% acetic acid and applied to a column of Aminex A-5. The column was then developed with gradients of pyridinium acetate constructed as described in Degen and Kyte (1978). The columns had dimensions of ether 0.3 × 8.3 cm or 0.9 × 10 cm, and the two gradients were each 100 mL with each column. Distributions of peptide were determined by alkaline ninhydrin (Hirs et al., 1956). Samples for HPLC were dissolved in either 0.1% trifluoroacetic acid or 40% formic acid and injected onto a Waters μBondapak C_{18} column (0.4 × 30 cm) or a Vydac C_4 column (0.46 × 25 cm). The HPLC system consisted of the following components from Waters Associates: two M6000A pumps, a UK6 injector, a 680 automated gradient controller, and a 440 UV detector equipped with an extended-wavelength module for detection of absorbance at 229 nm. The column was developed with gradients that were 0.1% (v/v) trifluoroacetic acid and variable concentrations of acetonitrile (Mahoney & Hermodson, 1980).

Reductive Amination with Pyridoxal Phosphate and [^{3}H]$NaBH_4$. The procedure used was adapted from that of Ohkawa and Webster (1981). Samples dissolved or suspended in 60 mM sodium borate, pH 8.0, are pretreated with 0.7 mM $NaBH_4$ to reduce any aldehydes or ketones that may be present. After 10 min, pyridoxal phosphate is added to 6 mM. After 15 min, a half-molar equivalent of [^{3}H]$NaBH_4$ (specific radioactivity 300–700 Ci mol^{-1}) is added to the solution, and the reduction is allowed to proceed for 20 min. In all subsequent operations, samples are protected from light to prevent loss of label.

In model reactions, samples (80 μL) of reduced and carboxamidomethylated bovine serum albumin (1–4 mg mL^{-1}) in various buffers between pH 7.0 and 8.0 were mixed with various amounts of 0.2 M pyridoxal phosphate (final concentrations 6–100 mM). After various times (2–20 min), various amounts (0.5–2 molar equivalents) of $NaBH_4$ in 0.01 M NaOH were added. After 10 min, the samples were applied to small columns of Sephadex G-50M that were rapidly centrifuged (Penefsky, 1977) to isolate the labeled protein. The amount of bound pyridoxamine was determined spectrophotometrically (Kirtley & Koshland, 1972).

When sealed, right-side-out vesicles were labeled, they were first transferred to 0.25 M sucrose and 60 mM sodium borate, pH 8.0. After reductive amination in the presence or absence of 0.4% saponin, vesicles to be treated with trypsin were collected by centrifugation for 30 min at 45 000 rpm in a Beckman Ti50.2 rotor. Vesicles from which the modified α polypeptide was to be isolated were dialyzed against several changes of 0.25 M sucrose, 1 mM EDTA, and 30 mM histidinium chloride, pH 7.4, and then collected by ultracentrifugation.

Antipeptide Antibodies. The peptide KGAPER was manually synthesized by using the solid-phase method of Merrifield (1963) as described by Stewart and Young (1969) with the

recent modifications (Merrifield et al., 1982). The initial amino acid, arginine, was attached to chloromethylstyrene beads as N^{α}-Boc-N^{γ}-(*p*-toluenesulfonyl)-L-arginine by reflux in absolute ethanol 3% in triethylamine for 65 h. During the synthesis, the following protected L-amino acids were used: O^{γ}-benzyl-N^{α}-Boc-L-glutamate, N^{α}-Boc-L-proline, N^{α}-Boc-L-alanine, N^{α}-Boc-L-glycine, and N^{ϵ}-(benzoxycarbonyl)-N^{α}-Boc-L-lysine. After synthesis, the peptide was cleaved from the resin using liquid hydrogen fluoride, washed in anhydrous ether and ethyl acetate, extracted in 5% acetic acid in water, and finally lyophilized. The peptide GAPER was produced from KGAPER by Edman degradation by the method of Hartley (1970), as described by Narita et al. (1975). The extent of the removal of lysine was followed by HPLC.

A conjugate of bovine serum albumin and the peptide, KGAPER, was prepared according to Walter et al. (1980). The peptide (10.2 μmol) was dissolved in a solution of bovine serum albumin (300 nmol) in 2 mL of 0.1 M sodium phosphate, pH 7.5. Glutaraldehyde (1 mL, 20 mM) was added and allowed to react for 30 min at room temperature. One-tenth volume of 1 M glycine was added to block glutaraldehyde that had not reacted, and the solution was then dialyzed against 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4 (phosphate-buffered saline).

This conjugate was used as antigen to raise antibodies in New Zealand white rabbits. Initial injection in complete Freund's adjuvant (1:1 emulsion with 1 mg mL^{-1} solution of antigen) was into foot pads, and boosting injections in incomplete Freund's adjuvant were subcutaneously administered in the back. Crude immunoglobulins were isolated from serum by precipitation with ammonium sulfate and stored at 4 °C as a slurry. They were redissolved and dialyzed into phosphate-buffered saline as needed.

To purify immunoglobulins specific for the carboxy-terminal sequence, -GAPER, an affinity column was made (March et al., 1974) from the synthetic peptide KGAPER and agarose beads (Sepharose 4B). Washed agarose beads (10 mL) were activated in 1 M sodium carbonate and 2.5% cyanogen bromide, and coupling was performed with 4 μmol of peptide in 0.2 M sodium bicarbonate for 20 h at 4 °C.

Crude immunoglobulins or serum was passed at room temperature over a column of this adsorbent, which was then washed extensively with phosphate-buffered saline and eluted with 0.1 M glycinium chloride, pH 2.5. The fractions containing eluted protein were pooled and dialyzed against phosphate-buffered saline and the affinity-purified immunoglobulins precipitated with ammonium sulfate. These immunoglobulins in turn were coupled to agarose beads activated with cyanogen bromide, to produce the immunoadsorbent specific for the carboxy-terminal sequence, -GAPER. The coupling was performed in phosphate-buffered saline, with activated beads that had been washed with this buffer, at 4 °C for 20 h; 1.5 mg (nominally 20 nmol) of immunoglobulin (mL of activated agarose)$^{-1}$ was present during the coupling reaction. After being quenched with glycine, at a final concentration of 0.3 M, for 5 h at 4 °C, the immunoadsorbent was washed with 1 M ammonium sulfate and phosphate-buffered saline. Supernatants from tryptic digests of right-side-out vesicles labeled with [^{3}H]pyridoxamine phosphate were passed directly over a column formed from this immunoadsorbent, which was washed with phosphate-buffered saline and eluted with 0.1 M sodium phosphate, pH 2.5.

RESULTS

Pyridoxal Phosphate Labeling Reaction. The impermeant reagent chosen for labeling sealed, right-side-out vesicles was

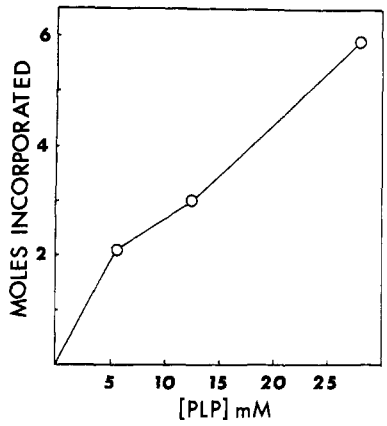

FIGURE 1: Incorporation of pyridoxal phosphate into carboxamidomethylated bovine serum albumin. Bovine serum albumin (4 mg mL^{-1}) in 60 mM sodium borate, pH 8.0, was incubated for 10 min with the noted final concentrations of pyridoxal phosphate (PLP) at room temperature. Sodium borohydrate (0.5 molar equivalent with respect to pyridoxal phosphate) was added as an 0.25 M solution in 10 mM NaOH. After 10 min, the entire sample was passed through a small column (1 mL) of Sephadex G-50 M by centrifugation. The amounts of pyridoxamine and bovine serum albumin in the eluates were determined spectrophotometrically. The ordinate is graduated in moles of pyridoxamine per mole of serum albumin.

pyridoxal phosphate, and the pyridoximinium phosphate adducts of lysine residues on the protein were reduced to [^{3}H]pyridoxamine phosphate with [^{3}H]$NaBH_4$. We have conducted extensive experiments to characterize the effects of pyridoxal phosphate concentration, sodium borohydride concentration, time, pH, and protein concentration upon the reaction. For most of these experiments, reduced and carboxamidomethylated bovine serum albumin was chosen as a model protein because it is denatured, which provides maximum access to lysine residues, yet also soluble under the conditions of the labeling reaction. The effect of increasing pyridoxal phosphate concentration upon the reductive alkylation of reduced and carboxamidomethylated bovine serum albumin was examined at a protein concentration and pH similar to those later used for labeling vesicles. There was a linear increase in the incorporation of pyridoxamine with increasing concentration of pyridoxal phosphate (Figure 1). At a concentration of 28 mM pyridoxal phosphate, about 0.1 nmol of pyridoxamine (nmol of lysine)$^{-1}$ was incorporated into reduced and carboxamidomethylated bovine serum as judged by spectral analysis of the product. For labeling vesicles, we used conditions very similar to those of Ohkawa and Webster (1981). A pyridoxal phosphate concentration of 6 mM was chosen to make the most efficient use of [^{3}H]$NaBH_4$ while producing adequate incorporation of radioactivity.

Sealed, Right-Side-Out Vesicles. Forbush (1982) has described a preparation of sealed, right-side-out vesicles containing high concentrations of (Na^+ + K^+)-ATPase. These vesicles have subsequently been used in modification experiments with the impermeant reagent diazotized *p*-[^{35}S]sulfanilic acid (O'Connell, 1982). They have been demonstrated to be right-side-out by several criteria (Forbush, 1982; O'Connell, 1982). One of the observations which demonstrated that they also are sealed, at least to MgATP, was the latency of the (Na^+ + K^+)-ATPase activity. When the detergent deoxycholate was added to a suspension of these vesicles, the activity of the enzyme increased dramatically (Figure 2), presumably the result of the access gained by the substrate to internal active sites. Saponin, a heterogeneous mixture of glycosides that do not dissolve biological membranes but do create holes in them wide enough to pass large protein molecules (St. John et al.,

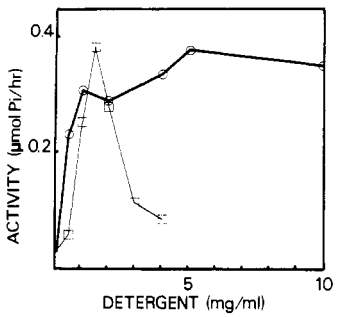

FIGURE 2: Effect of saponin and deoxycholate on $(Na^+ + K^+)$-ATPase activity in sealed, right-side-out vesicles. Sealed vesicles (0.2 mg mL^{-1}) were exposed for 12 min at room temperature to various concentrations of either deoxycholate (□) or saponin (○). Portions were then removed from the samples and assayed for $(Na^+ + K^+)$-ATPase activity.

1982), also breached the sealed, right-side-out vesicles. In the case of saponin, however, we have always observed only a monotonic increase in enzymatic activity followed by a constant maximum as the concentration of the saponin is increased (Figure 2) with never any indication of a sharp maximum such as the one seen in the presence of deoxycholate. The maximum level maintained in the presence of saponin is never less than that seen at the optimal concentration of deoxycholate. This suggests that the maximum maintained at the high concentrations of saponin is the maximum possible for the amount of enzyme present and that saponin does not inhibit enzymatic activity.

Saponin has two important advantages over a detergent like deoxycholate. The membranes should remain intact after treatment with saponin (St. John et al., 1982) so that the same bilayer should still surround each molecule of $(Na^+ + K^+)$-ATPase and the steric accessibility of amino side chains near or within the bilayer should not change. Enzymatic activity is not lost at higher concentrations of saponin, as it is at higher concentrations of deoxycholate (Figure 2), so that changes in the accessibility of amino acid side chains due to denaturation will not affect the results. From these considerations, it can be concluded that the only difference between a molecule of $(Na^+ + K^+)$-ATPase in a sealed, right-side-out vesicle and one in a vesicle that has been exposed to saponin is the access of impermeant reagents to its cytoplasmic surface.

Sealed, right-side-out vesicles that had been labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ were dissolved and submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis. A dramatic increase in the incorporation of radioactivity into the α polypeptide of $(Na^+ + K^+)$-ATPase was observed when saponin had been added to the vesicles prior to the addition of the pyridoxal phosphate (Figure 3). Labeling of other proteins (mostly located to the right of the α polypeptide in Figure 3) was not so significantly affected by the saponin. In the absence of pyridoxal phosphate, little radioactivity was incorporated into any of the proteins (Figure 3).

Sealed, right-side-out vesicles that had been labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ in the presence of saponin were dissolved, and the α polypeptide of $(Na^+ + K^+)$-ATPase was partially purified from them by gel filtration in 0.1% $NaDodSO_4$ (Kyte, 1972). From the protein concentration and the tritium content (cpm) of the pooled material, as well as the specific radioactivity of the initial $[^3H]NaBH_4$, it can be calculated that 0.02 nmol of tritium was incorporated for every nanomole of lysine in the sample. This can be compared to the 0.03 nmol of pyridoxamine (nmol of lysine)$^{-1}$ that was incorporated into reduced and carboxamidomethylated bovine serum albumin at 6 mM pyridoxal phosphate (Figure 1).

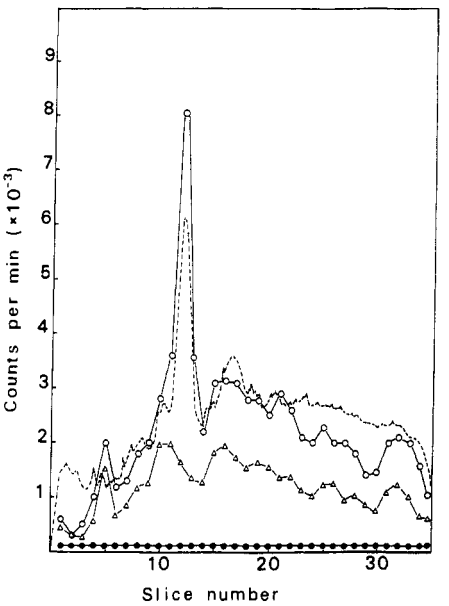

FIGURE 3: Changes in the incorporation of [^{3}H]pyridoxamine phosphate into the α polypeptide of $(Na^+ + K^+)$-ATPase in sealed, right-side-out vesicles caused by the addition of saponin. Sealed, right-side-out vesicles (5.5 mg mL^{-1}) were mixed with control buffer (▲) or a 7% solution of saponin (○) such that the final concentration of saponin was 0.4%. After 15 min, the vesicles were labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$. A third sample that received neither saponin nor pyridoxal phosphate (●) was also carried through each step. The vesicles were collected by ultracentrifugation, resuspended, and dissolved with $NaDodSO_4$, and portions (100 μg) from each sample were submitted to electrophoresis on 5% polyacrylamide gels. The gels were siliced, and the distribution of tritium was determined. The mobility of the large peak of radioactivity in slice 12 corresponds to that of the α polypeptide of $(Na^+ + K^+)$-ATPase, which can be seen in a scan of a stained gel of these vesicles (- - -).

Distribution of [^{3}H]Pyridoxamine Phosphate over the α Polypeptide. Sealed, right-side-out vesicles were labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ in the presence or absence of 0.4% saponin, the α polypeptide of $(Na^+ + K^+)$-ATPase was purified from them by gel filtration, and it was digested with thermolysin. When these digests were submitted to cation-exchange chromatography, it was observed that the number of radioactive peptides increased significantly when saponin had been added to the original vesicles (Figure 4). Since many peptides had been labeled in the presence of saponin, this result indicates that many of the lysines in the α polypeptide of native $(Na^+ + K^+)$-ATPase react with pyridoxal phosphate when the vesicles have been opened.

The promiscuity of the pyridoxal phosphate in its reaction with lysine residues on the proteins in sealed, right-side-out vesicles could also be demonstrated by directly digesting vesicles that had been labeled with pyridoxal phosphate and $[^3H]NaBH_4$ in the presence of saponin. Six major peptides were released from the membranes by this digestion (Figure 5A), which was performed in the presence of 0.5% saponin. At the same time, four major radioactive peptides were also released (Figure 5C). Although no direct connection can be made between nonradioactive and radioactive peptides because the pyridoxamine phosphate must have blocked tryptic digestion at modified lysines, these results suggest that a majority of the peptides released from the native membranes by trypsin contained lysine residues that could be modified by pyridoxal phosphate and $[^3H]NaBH_4$. The results of this experiment also demonstrated that trypsin released a similar collection of nonradioactive peptides from vesicles labeled in the presence

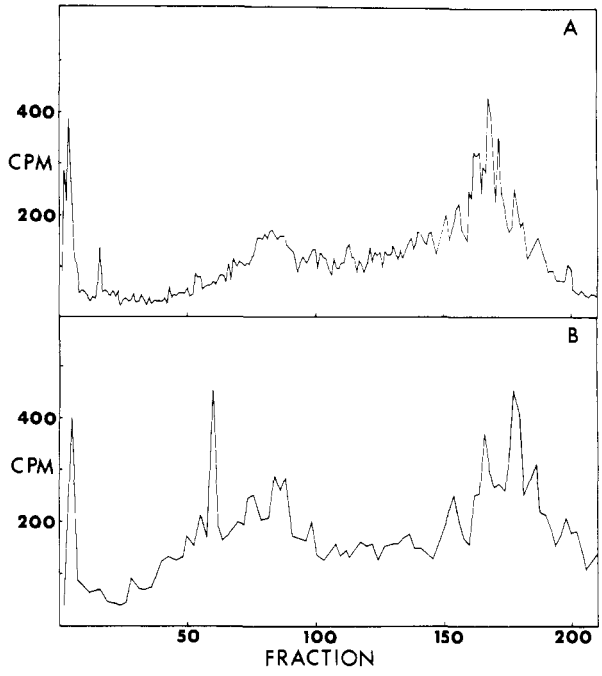

FIGURE 4: Cation-exchange chromatography of thermolytic peptides of the α polypeptide of $(Na^+ + K^+)$-ATPase from sealed, right-side-out vesicles labeled in the absence (A) or presence (B) of saponin. Sealed vesicles (4 mg mL^{-1}) were labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ in the absence or presence of 0.4% saponin. After dialysis, the vesicles were collected by centrifugation and dissolved with $NaDodSO_4$, and the α polypeptide was partially purified by gel filtration in 0.1% $NaDodSO_4$. After removal of the dodecyl sulfate, each sample was digested with thermolysin, lyophilized, redissolved in 60% acetic acid, and loaded onto a column (0.3 × 8.3 cm) of Aminex A-5 resin. The column was developed with consecutive gradients of pyridinium acetate. Counts per minute (cpm) of tritium were determined in portions (25 μL) from each fraction (0.75 mL).

(Figure 5A) or the absence (Figure 5B) of saponin.

Immunoadsorbent Specific for the Carboxy-Terminal Sequence, -GAPER. As an initial step toward determining the location of Lys_{501}, found in the tryptic peptide HLLVMKGAPER, within the α polypeptide of intact, canine $(Na^+ + K^+)$-ATPase (Farley et al., 1984), the peptide KGAPER was synthesized by solid-phase techniques (Merrifield et al., 1982). The synthetic peptide was purified by cation-exchange chromatography, and the purified peptide was chromatographically homogeneous upon both cation-exchange chromatography and reverse-phase HPLC. Amino acid analysis of the purified peptide gave the following composition: $K_{0.99}G_{0.98}A_{1.02}P_{1.02}E_{1.00}R_{0.99}$. The purified peptide was coupled to bovine serum albumin with glutaraldehyde (Walter et al., 1980). The molar ratio of covalently attached peptide to bovine serum albumin was determined to be 15 by amino acid analysis. This conjugate was injected into rabbits to produce polyclonal antibodies specific for the carboxy-terminal sequence, -GAPER.

To purify these specific immunoglobulins, an affinity column was made from the synthetic peptide KGAPER and agarose beads by coupling with cyanogen bromide (March et al., 1974). To determine the concentration of accessible carboxy termini attached to the beads, a sample was submitted to carboxypeptidase digestion during which 180 nmol of arginine (mL of agarose)$^{-1}$ was released. Crude immunoglobulins were passed over this affinity resin, and immunoglobulins specific for -GAPER were eluted with acid.

The purified immunoglobulins were then attached to agarose beads by cyanogen bromide activation to produce an immunoadsorbent. The capacities of the immunoadsorbents were determined by saturating them with synthetic peptide, rinsing extensively, releasing the bound peptide with acid, and determining by amino acid analysis the amount of peptide released. The capacity of the immunoadsorbent used for most of these experiments was 4.5 nmol of peptide (mL of settled immunoadsorbent)$^{-1}$. The same immunoadsorbent could be reused repeatedly.

Determination of the Location of Lys_{501} in Intact $(Na^+ + K^+)$-ATPase. Sealed, right-side-out vesicles (30 mg in 5.5 mL of each sample), whose enzymatic activity was enhanced 24-fold upon addition of saponin (final specific enzymatic activity of 400 μmol mg^{-1} h^{-1}), were labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ in the absence or presence of 0.4% saponin. Saponin caused the usual increase (Figure 3) in the incorporation of tritium into the α polypeptide of $(Na^+ + K^+)$-ATPase when this was checked by $NaDodSO_4$–polyacrylamide gel electrphoresis. Both samples were then digested with trypsin (1:10 by weight) after the addition of saponin to 0.5% to render the vesicles permeable to the proteolytic enzyme. Tryptic digestion of modified, native, canine $(Na^+ + K^+)$-ATPase, in open membrane fragments in the absence of saponin, has been shown to release the modified peptide $_{496}$HLLVMK(X)GAPER$_{506}$ (Farley et al., 1984), where X indicates the modification.

Tryptic digests of the vesicles labeled with $[^3H]$pyridoxamine phosphate were passed over a column containing the immunoadsorbent directed against the carboxy-terminal sequence, -GAPER, and bound tritium was eluted in each case with acid (Figure 6). As a control, to determine what fraction of the bound and eluted tritium was attached to tryptic peptides with the carboxy-terminal sequence, -GAPER, equivalent samples of each digest were mixed with a large molar excess of the synthetic peptide KGAPER before immunoadsorption. Very little, if any, tritium was adsorbed and eluted in the control experiments (Figure 6A,B), and it can be concluded that the majority of the tritium eluted with acid in the experimental samples was attached to a peptide or peptides with the carboxy-terminal sequence, -GAPER.

When the digest of the vesicles that had been labeled in the presence of saponin was submitted to immunoadsorption, at least 5-fold more tritium was bound and eluted with acid than in the case of the digest of vesicles labeled in its absence (Figure 6C). Fewer cpm of tritium were added to the immunoadsorbent in the case of digests from the vesicles labeled in the absence of saponin (inset, Figure 6C) because the overall incorporation of tritium into peptides released in this digest was less than the incorporation into the peptides released upon digestion of vesicles labeled in the presence of saponin (Figure 5C) even though the same quantity of nonradioactive peptides was released in each digest (Figure 5A,B).

The peptides in the acid eluates from the digests of vesicles labeled either in the presence or in the absence of saponin (fractions 16, 17, and 18 in Figure 6C) were collected, concentrated by evaporation, redissolved, and submitted to HPLC (Figure 7). Both eluates from the immunoadsorbent contained the same amount (Figure 7C,D) of a peptide that had a mobility very similar to the peptide GAPER (Figure 7B), produced by Edman degradation (Hartley, 1970) of the synthetic peptide KGAPER (Figure 7A). These peptides from the digests of vesicles labeled either in the presence (Figure 7C) or in the absence (Figure 7D) of saponin, respectively, coeluted upon chromatography (Figure 7E). These results demonstrate that trypsin released approximately the same amount of GAPER from vesicles modified either in the presence or in the absence of saponin (Figure 7C,D) even

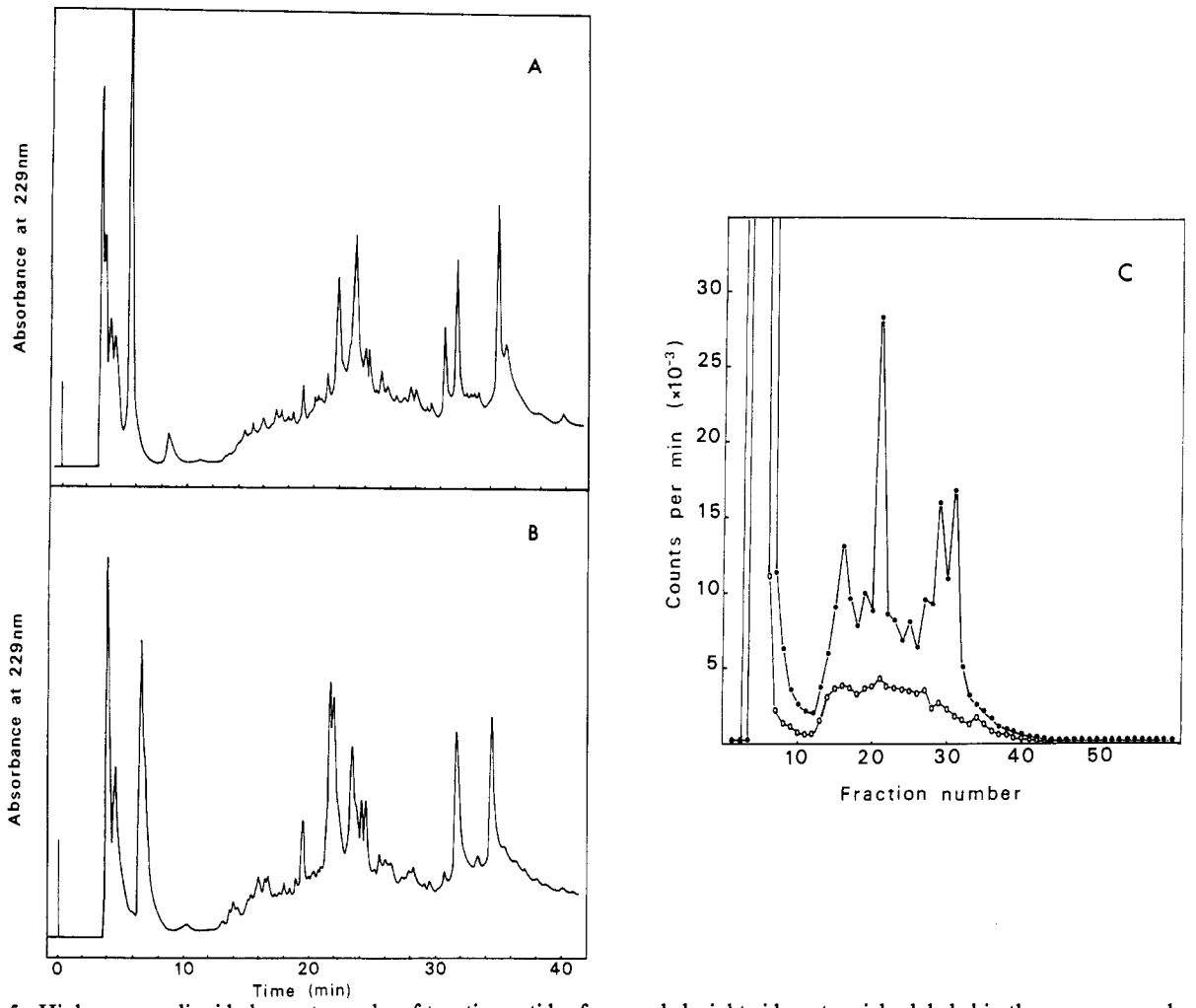

FIGURE 5: High-pressure liquid chromatography of tryptic peptides from sealed, right-side-out vesicles labeled in the presence or absence of saponin. Sealed vesicles (5.5 mg mL^{-1}) were labeled with 6 mM pyridoxal phosphate and $[^3H]NaBH_4$ in the absence or presence of 0.5% saponin. The vesicles in both samples were washed by ultracentrifugation, resuspended in 0.5% saponin, 1 mM EDTA, and 25 mM imidazolium chloride, pH 7.5, and digested with trypsin. The remaining particulate material was removed by ultracentrifugation; portions of the supernatants were lyophilized, redissolved in 0.1% trifluoroacetic acid, and injected onto a C_4 reverse-phase column that was then developed with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} over 60 min. Continuous monitoring of the effluent at 229 nm was performed, and fractions (1 mL) were collected and their tritium content determined by liquid scintillation. (A) Vesicles labeled in the presence of saponin. (B) Vesicles labeled in the absence of saponin. (C) Distribution of tritium in chromatograms of digests from vesicles labeled in the absence (○) or presence (●) of saponin.

though the amounts of tritium released by trypsin and bound and eluted from the immunoadsorbent, presumably as the peptide HLLVMK([^{3}H]pyridoxamine phosphate)GAPER, were remarkably different (Figure 6C).

The nanomoles of GAPER bound from each sample by the immunoadsorbent and released from the immunoadsorbent by acid can be estimated from the absorbance of the peaks in the chromatograms (Figure 7). Each of the peaks corresponding to GAPER in Figure 7C,D contained 0.4 nmol of the peptide; that in Figure 7E, 1.0 nmol. The nanomoles of $(Na^+ + K^+)$-ATPase in each sample of the original sealed, right-side-out vesicles can be estimated from its total $(Na^+ + K^+)$-ATPase activity and the turnover number of canine $(Na^+ + K^+)$-ATPase in our assay, 270 μmol of P_i (nmol of enzyme·h)$^{-1}$, determined by a titration of ouabain binding sites (Fortes, 1977). Each original sample of vesicles contained 46 nmol of $(Na^+ + K^+)$-ATPase. From these two values, it can be estimated that at least 0.63 nmol of GAPER was released by trypsin for every nanomole of $(Na^+ + K^+)$-ATPase in the initial vesicles. Considering the number of manipulations required, this is an appropriate yield. From the specific radioactivity of the $[^3H]NaBH_4$ (320 Ci mol^{-1}), the tritium bound and eluted (19 200 cpm) during the immunoadsorption of the digest from vesicles labeled in the presence of saponin (Figure 6A), and the amount of GAPER bound and released (1.3 nmol) from the same digest (Figure 7), it can be calculated that 0.05 nmol of [^{3}H]pyridoxamine phosphate incorporated into Lys_{501} was released by trypsin for every nanomole of GAPER released by trypsin.

Discussion

If it is assumed that both the release of the peptide GAPER and the release of tritium, presumably as the peptide HLLVMK([^{3}H]pyridoxamine phosphate)GAPER, by trypsin from the labeled vesicles occurred with equal efficiency, it can be concluded that the maximum yield of the reductive amination at Lys_{501} under the conditions chosen for the reaction was 5%. Therefore, most of the molecules of $(Na^+ + K^+)$-ATPase in either the vesicles modified in the presence of saponin or the vesicles modified in its absence were unmodified at Lys_{501}, and during the subsequent tryptic digestion, the same amounts of GAPER should have been released from each sample. The fact that the yields of GAPER from each were the same (Figure 7C,D) demonstrates that the tryptic digestion, immunoadsorption, and elution occurred with equal efficiency during the processing of the two samples. It may

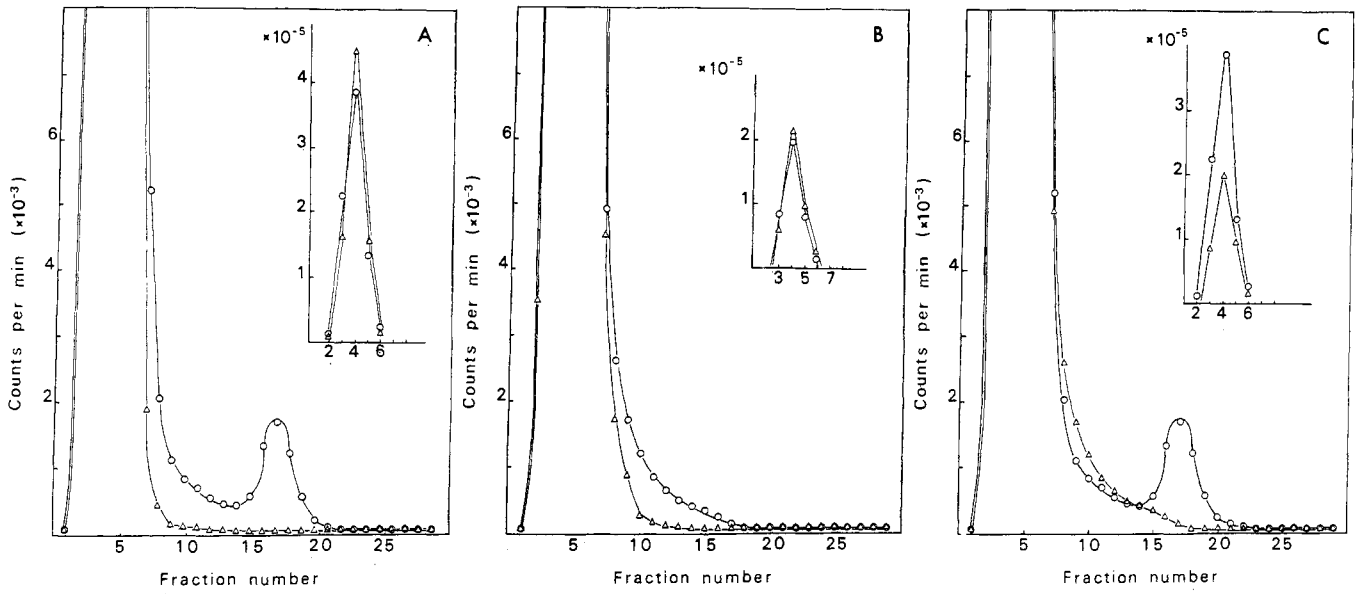

FIGURE 6: Immunoaffinity chromatography of tryptic digests of sealed and unsealed, right-side-out vesicles on a column of immobilized anti-GAPER antibody. Supernatants of tryptic digests were either added directly to a column (2.5 mL) of agarose beads to which anti-GAPER immunoglobulin had been attached (11-nmol capacity) or added after they had been mixed with 200 nmol of the synthetic peptide, KGAPER. The column was then washed with 14 mL of phosphate-buffered saline (fractions 1–14) and eluted with 0.1 M sodium phosphate, pH 2.5 (fractions ≥15). Portions (0.3 mL) of each fraction (1.0 mL) were submitted to liquid scintillation. Insets: Counts per minute from initial fractions plotted on expanded scales. (A) Digests of vesicles labeled in the presence of 0.4% saponin added directly (○) to immunoadsorbent or after addition of 200 nmol of KGAPER (△). (B) Digests of vesicles labeled in the absence of saponin added directly (○) or after addition of 200 nmol of KGAPER (△). (C) Replot of traces from the directly added samples from A (○) and B (△) for comparison. In all cases, the same relative amount of each digest based on the protein concentration in the original sample of vesicles was added to the column.

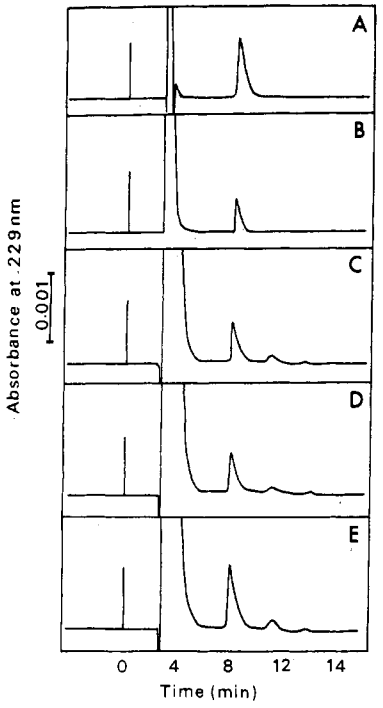

FIGURE 7: High-pressure liquid chromatography of peptides bound and released from the immunoadsorbent in the experiment displayed in Figure 6. The remainders (0.7 mL) of fractions 16, 17, and 18 (Figure 6C), containing peptides from the digests of vesicles labeled in the absence or presence of saponin, were combined, evaporated to dryness, and redissolved in 0.7 mL of 0.1% trifluoroacetic acid. Portions (0.35 mL) were injected onto a C_{18} reverse-phase column that was eluted with a linear gradient of 0–10% acetonitrile over 30 min. (A) Synthetic KGAPER. (B) Synthetic KGAPER submitted to one cycle of Edman degradation. (C) Sample from digest of vesicles that had been labeled in the presence of saponin. (D) Sample from digest that had been labeled in the absence of saponin. (E) Mixture of the two samples applied in (C) and (D).

be concluded that differences in the amount of tritium eluted during the immunoadsorptions performed on the two samples (Figure 6C) reflect real differences in the extent of incorporation into Lys_{501} during the reductive amination.

When sealed, right-side-out vesicles were treated with saponin, the Lys_{501} in the $(Na^+ + K^+)$-ATPase they contain became susceptible to reductive amination by pyridoxal phosphate and $[^3H]NaBH_4$. The most reasonable explanation for this increase in reactivity is that Lys_{501} residues on the inside of the vesicle on the cytoplasmic surface of $(Na^+ + K^+)$-ATPase.

It is already thought that Lys_{501} is located on the cytoplasmic surface of $(Na^+ + K^+)$-ATPase. This belief, in part, results from the fact that Lys_{501} is modified by fluorescein 5′-isothiocyanate in a reaction prevented from occurring by the addition of ATP (Karlish, 1980). It has been proposed that this result means that Lys_{501} is in the vicinity of the active site at which MgATP is hydrolyzed (Farley et al., 1984) and hence on the cytoplasmic surface (Whittam, 1962; Sen & Post, 1964). This argument ignores the large global conformational changes that occur in $(Na^+ + K^+)$-ATPase during the steps of the enzymatic reaction. For example, dephosphorylation of the aspartyl phosphate at the enzymatic active site lowers the affinity of the enzyme for ouabain by greater than 50-fold (Wallick & Schwartz, 1974) even though the active site and the binding site for ouabain are on opposite sides of the bilayer. The protection of the enzyme by ATP from modification by fluorescein 5′-isothiocyanate could easily be such an allosteric effect. Formally, the binding of ATP on the cytoplasmic side of the enzyme could alter the nucleophilicity of Lys_{501} even if it were located on the extracytoplasmic side. In fact, large changes in the fluorescence of the covalently bound fluorescein are caused by the binding of ouabain to the extracytoplasmic surface of the enzyme (Karlish, 1980; Hegyvary & Jørgensen, 1981).

The other argument that has been put forth to locate Lys_{501} on the cytoplasmic side of $(Na^+ + K^+)$-ATPase is that there are no obvious membrane-spanning sequences between it and Asp_{369} which lies within the active site for the hydrolysis of

MgATP. Whether or not membrane-spanning sequences can be reliably identified, however, by scanning the sequence of a protein for hydrophobic segments (Kyte & Doolittle, 1982) has not been established. It is possible that membrane-spanning sequences do lie between Asp_{369} and Lys_{501}. Since it has now been demonstrated that Lys_{501} is located on the cytoplasmic surface of $(Na^+ + K^+)$-ATPase, as is Asp_{369}, there must be no membrane-spanning sequences between these two residues or an even number of membrane-spanning sequences.

Pyridoxal phosphate, a stable, water-soluble aldehyde, reacts reversibly with amino groups, such as lysyl residues in a protein, to yield an imine. The iminium cation can be rapidly reduced with $NaBH_4$ to produce a stable N-substituted pyridoxamine phosphate. This secondary amine can be readily rendered radioactive by using either $[^3H]NaBH_4$ or isotopically labeled pyridoxal phosphate. Pyridoxal phosphate has been used as an impermeant reagent to label proteins exposed to the exterior surface of sealed, membrane systems (Rifkin et al., 1972; Hunt & Brown, 1974; Cabantchik et al., 1975; Juliano & Behar-Bannelier, 1975; Golds & Braun, 1976; Garadi & Babitch, 1980; Ohkawa & Webster, 1981; Bogner et al., 1982). It has been used as a probe both to determine which of a collection of proteins are exposed on the exterior surface of the membrane and, at a finer level, to determine whether particular lysyl residues in a given protein are exposed or not.

At the concentration of pyridoxal phosphate that we have chosen for labeling $(Na^+ + K^+)$-ATPase, 6 mM, the extent of incorporation was low—in each enzyme molecule, an average of only 2% of the lysines were modified. Such a low level of modification has been stated to be necessary to avoid gross alterations of the structures of either the protein or the membrane (Bretscher, 1971). A disadvantage of working at such low levels of incorporation is that an unrepresentative population of particularly reactive lysines may account for a large part of the label incorporated. In our situation, denaturation of the protein could have increased dramatically the nucleophilicity of the lysine residues, and the changes in levels of incorporation observed would only have been a reflection of differences in levels of denaturation. In fact, under some circumstances, pyridoxal phosphate has been selective. At concentrations less than 200 μM, only particular lysines in some proteins are labeled. Aspartate transcarbamoylase (Greenwell et al., 1973) and 6-phosphogluconic dehydrogenase (Rippa et al., 1967) were both inactivated upon the incorporation of 1 mol of pyridoxamine phosphate for every mole of active sites.

Under the labeling conditions we have chosen, however, with much higher levels of pyridoxal phosphate, a large number of lysines were reacting. The linear increase of incorporation into carboxamidomethylated bovine serum albumin with increasing concentrations of pyridoxal phosphate (Figure 1) implies that the majority of the lysines in this protein were reacting, each in fairly low yield, each with about the same nucleophilicity. The level of incorporation of $[^3H]$pyridoxamine phosphate into the total lysines in partially purified α polypeptide from vesicles labeled in the presence of saponin [0.02 nmol of ^3H (nmol of lysine)$^{-1}$] and that into Lys_{501} itself [0.05 nmol of ^3H (nmol of $(Na^+ + K^+)$-ATPase)$^{-1}$] compare favorably with that into the total lysines of denatured bovine serum albumin under the same conditions [0.03 nmol of pyridoxamine (nmol of lysine)$^{-1}$]. These results indicate that the reaction of pyridoxal phosphate with the lysines in $(Na^+ + K^+)$-ATPase was also a nonselective one. Chromatographic profiles of digested, labeled $(Na^+ + K^+)$-ATPase (Figures 4 and 5) show a large number of radioactive peptides. Using similar conditions to those that we have used, Bogner et al. (1982) were able to isolate peptides containing pyridoxamine phosphate from 35% of the lysines in bovine mitochondrial ATP–ADP carrier. All of these results strongly suggest that the incorporation of $[^3H]$pyridoxamine phosphate observed in the present experiments was into native $(Na^+ + K^+)$-ATPase, many of whose lysines were reacting quite freely with the aldehyde.

Various experimental results demonstrate that the vesicles used in these experiments are sealed to impermeant reagents, including pyridoxal phosphate. The $(Na^+ + K^+)$-ATPase activity increased up to 20-fold when they were treated with saponin (Figure 2) due to the inability of the external substrates, MgATP and Na^+, to gain access to the active site prior to the treatment with surfactant. The incorporation of both diazotized $[^{35}S]$sulfanilic acid (O'Connell, 1982) and $[^3H]$-pyridoxamine phosphate (Figure 3) into the α polypeptide of $(Na^+ + K^+)$-ATPase increased about 3–4-fold upon treatment of these vesicles with surfactants that render them permeable. The similarity between these two results, one set obtained with a zwitterionic reagent and the other with an anionic reagent, suggests that in both cases the increase in incorporation was due to the same process, namely, the opening of vesicles permeable to both reagents before treatment with the surfactants. In experiments performed by Dzhandzhugazyan and Jørgensen (1985), it was also observed that the incorporation of an impermeant reagent, *N*-succinimidyl 3-(4-hydroxy-5-$[^{125}I]$iodophenyl)propionate, into the α subunit of native $(Na^+ + K^+)$-ATPase increased 3–4-fold when the modification of sealed vesicles and that of open fragments of membrane, both from porcine kidneys, were compared. The agreement of these ratios between the incorporation into sealed and opened vesicles with similar ratios obtained in experiments with vesicles of reconstituted $(Na^+ + K^+)$-ATPase (O'Connell, 1982) and intact cells (Sharkey, 1983) suggests that the ratios reflect only the inherent ratio of the distribution of mass of the α polypeptide across the membrane (Nicholas, 1984) and that the vesicles used here were sealed and opened as designated. These vesicles float upon a concentrated solution of the dense salt 1-deoxy-1-(methylammonium)-D-glucitol 3,5-(diacetamido)-2,4,6-triiodobenzoate (Forbush, 1982) presumably because they are impermeable to it. They have been used extensively in studies of cation transport (Forbush, 1984a, 1986b), which required that they be tightly sealed to small cations such as Na^+ and K^+.

That these vesicles are exclusively right-side-out has been demonstrated previously. Trypsin, which only cleaves $(Na^+ + K^+)$-ATPase at its cytoplasmic surface (Giotta, 1975; Karlish & Pick, 1981; Chin & Forgac, 1983), will only cleave the α polypeptide of $(Na^+ + K^+)$-ATPase in these vesicles when they have been opened (Forbush, 1982; O'Connell, 1982). Their behavior in the vectorial transport of Na^+ and K^+ in the presence of MgATP is also fully consistent with a right-side-out orientation (Forbush, 1984a,b).

An alternative explanation for the increase in the incorporation of $[^3H]$pyridoxamine phosphate into $(Na^+ + K^+)$-ATPase observed here is that saponin not only allows access to the cytoplasmic face of the enzyme but also alters its structure as well. The fact that enzymatic activity was preserved in the presence of high concentrations of saponin (Figure 2) argues strongly against this, because alterations in the native structure of the enzyme would likely have resulted in loss of enzymatic activity.

These experiments illustrate a more general immunochemical strategy for determining the chemical reactivity of a specific amino acid residue in a protein. The strategy relies on a knowledge of the sequence of the protein of interest, but today, sequences are usually known before a protein becomes available for chemical studies. The strategy was prompted by the recently renewed interest in antipeptide antibodies (Walter et al., 1980) and by the ideas and methods of Wilchek et al. (1971) for immunochemically purifying modified peptides from the digest of a modified protein.

The key to the strategy employed here is the design of the synthetic peptide that will be used to elicit an antibody capable of recognizing the amino-terminal or carboxyl-terminal sequence of an indigenous peptide, a peptide derived from the intact polypeptide by a specific digestion and containing the targeted amino acid of interest. The sequence of the protein around the targeted amino acid is examined to discover a segment that will yield a small, water-soluble peptide upon digestion of the intact protein by chemical or enzymatic cleavages. The indigenous peptide chosen must be long enough so that the targeted amino acid will be flanked on one side by at least three or four amino acids that will not be altered by the modifications to be studied. The flanking amino acids serve as a handle upon the targeted amino acid and permit the indigenous peptide derived from the intact protein by digestion to be recognized by the antibody. This flanking sequence is incorporated as the amino-terminal or carboxy-terminal sequence of the synthetic peptide used as the hapten. A particularly critical feature of the flanking sequence on which the synthetic peptide is based is that it begin or end with the amino terminal or carboxy terminal, respectively, produced by the digestion yielding the indigenous peptide. Antibodies produced against synthetic peptide–haptens are probably directed mainly against their termini, which are the most accessible parts and the most peculiar structural features of the haptens.

Another aspect of the design of the synthetic peptide is that the targeted amino acid should not appear unaltered within the hapten because this would direct the antibody against the unmodified peptide in preference to the modified peptide. In the present experiments, we have used the position in the sequence that is occupied by the targeted amino acid as the point of attachment between the synthetic peptide–hapten and the carrier. This consideration is reiterated to the choice of a different coupling reaction with the same side chain for the production of the immunoadsorbent made from the peptide and used to purify the antibodies. Because the nature of the modification is never specified nor incorporated into the design, any reagent that might be used to modify the targeted amino acid can be assessed with the same immunoadsorbent.

The most significant drawback of this strategy is that the modified, indigenous peptide, as released by the digestion, must be soluble in an aqueous solution of moderate ionic strength buffered at neutral pH. This is an often neglected, but inescapable, requirement of immunoadsorption.

ACKNOWLEDGMENTS

We thank Dr. George Fortes for assisting us in the active-site titrations with anthroylouabain, Stephen Smith for supervision of the technical aspects of the experiments, Katherine Charles for preparation of microsomes, and Dennis Olshefski for performing the HF cleavage at the Peptide–Oligonucleotide Facility of the University of California at San Diego.

REFERENCES

Argos, P., Rao, J. K. M., & Hargrave, P. A. (1982) *Eur. J. Biochem. 128*, 565–575.

Bayley, H., & Knowles, J. R. (1980) *Biochemistry 19*, 3883–3892.

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

Bogner, W., Aguila, H., & Klingenberg, M. (1982) *FEBS Lett. 146*, 259–261.

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

Burgess, R. R. (1969) *J. Biol. Chem. 244*, 6168–6176.

Cabantchik, I. Z., Balshin, M., Breuer, W., & Rothstein, A. (1975) *J. Biol. Chem. 250*, 5130–5136.

Chin, G., & Forgac, M. (1983) *Biochemistry 22*, 3405–3410.

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

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature (London) 318*, 618–624.

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

Dzhandzhugazyan, K. N., & Jørgensen, P. L. (1985) *Biochim. Biophys. Acta 817*, 165–173.

Eisenberg, D., Schwartz, E., Komaromy, M., & Wall, R. (1984) *J. Mol. Biol. 179*, 125–142.

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.

Forbush, B. (1984a) *Anal. Biochem. 140*, 495–505.

Forbush, B. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5310–5314.

Fortes, P. A. G. (1977) *Biochemistry 16*, 531–540.

Garadi, R., & Babitch, J. A. (1980) *Biochim. Biophys. Acta 595*, 31–40.

Giotta, G. J. (1975) *J. Biol. Chem. 250*, 5159–5164.

Golds, E. E., & Braun, P. E. (1976) *J. Biol. Chem. 251*, 4729–4735.

Greenwell, P., Jewett, S. C., & Stark, G. R. (1973) *J. Biol. Chem. 248*, 5994–6001.

Guy, H. R. (1984) *Biophys. J. 45*, 249–261.

Hartley, B. S. (1970) *Biochem. J. 119*, 805.

Hegyvary, C., & Jørgensen, P. L. (1981) *J. Biol. Chem. 256*, 6296–6303.

Herrera, V. L., Emanuel, J. R., Ruiz-Opazo, N., Levenson, R., & Nadal-Ginard, B. (1987) *J. Cell Biol.* (in press).

Hirs, C. H. W., Moore, S., & Stein, W. H. (1956) *J. Biol. Chem. 219*, 623–642.

Hunt, R. C., & Brown, J. C. (1974) *Biochemistry 13*, 22–28.

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

Jørgensen, P. L., & Peterson, J. (1985) *Biochim. Biophys. Acta 821*, 319–333.

Juliano, R. L., & Behar-Bannelier, M. (1975) *Biochim. Biophys. Acta 375*, 249–267.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.

Karlish, S. J. D., & Pick, U. (1981) *J. Physiol. (London) 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 (London) 316*, 733–736.

Kirtley, M. E., & Koshland, D. E. (1972) *Methods Enzymol. 26*, 578–601.

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. (1981) *Nature (London) 292*, 201-204.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105-132.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199-11203.
Malatesta, F., Darley-Usmar, V., de Jong, C., Prochaska, L. J., Bisson, R., Capaldi, R. A., Steffens, G. C. M., & Buse, G. (1983) *Biochemistry 22*, 4405-4411.
March, S. C., Parikh, I., & Cuatracasas, P. (1974) *Anal. Biochem. 60*, 149-152.
Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149-2154.
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.
Narita, K., Matsuo, H., & Nakajima, T. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) pp 30-103, Springer-Verlag, New York.
Nicholas, R. N. (1984) *Biochemistry 23*, 888-898.
O'Connell, M. A. (1982) *Biochemistry 21*, 5984-5991.
Ohkawa, I., & Webster, R. E. (1981) *J. Biol. Chem. 256*, 9951-9958.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Ratnam, M., Nguyen, D. L., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986) *Biochemistry 25*, 2633-2643.
Rifkin, P. B., Compans, R. W., & Reich, E. (1972) *J. Biol. Chem. 247*, 6432-6437.
Rippa, M., Spanio, L., & Pontremoli, S. (1967) *Arch. Biochem. Biophys. 118*, 48-57.
Ross, A. H., Radhakrishnan, R., Robson, R. J., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 4152-4161.
Sen, A. K., & Post, R. L. (1964) *J. Biol. Chem. 239*, 345-352.
Shapiro, A. L., Viñuela, E., & Maizel, J. V., Jr. (1967) *Biochem. Biophys. Res. Commun. 28*, 815-820.
Sharkey, R. G. (1983) *Biochim. Biophys. Acta 730*, 327-341.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691-695.
Shull, G. E., Greeb, J., & Lingrel, J. B. (1986) *Biochemistry 25*, 8125-8132.
Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, pp 41-44, W. H. Freeman, San Francisco, CA.
St. John, P. A., Froehner, S. C., Goodenough, D. A., & Cohen, J. B. (1982) *J. Cell Biol. 92*, 333-342.
Wallick, E. T., & Schwartz, A. (1974) *J. Biol. Chem. 249*, 5141-5147.
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.
Whittam, R. (1962) *Biochem. J. 84*, 110-118.
Wilchek, M., Bocchini, V., Becker, M., & Givol, D. (1971) *Biochemistry 10*, 2828-2834.
Williams, J. C., Steiner, L. A., Feher, G., & Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7303-7307.
Winslow, J. (1981) *J. Biol. Chem. 256*, 9522-9531.

Phosphoenzyme Decomposition in Dog Cardiac Sarcoplasmic Reticulum Ca^{2+}-ATPase†

Taitzer Wang

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575

Received July 15, 1987; Revised Manuscript Received August 26, 1987

ABSTRACT: A five-syringe quench-flow apparatus was used in the transient-state kinetic study of intermediary phosphoenzyme (EP) decomposition in a Triton X-100 purified dog cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase at 20 °C. Phosphorylation of the enzyme by ATP in the presence of 100 mM K^+ for 116 ms gave 32% ADP-sensitive E_1P, 52% ADP- and K^+-reactive E_2P, and 16% unreactive residual EP_r. The EP underwent a monomeric, sequential $E_1P \xrightarrow{17\ s^{-1}} E_2P \xrightarrow{10.5\ s^{-1}} E_2 + P_i$ transformation and decomposition in the ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid quenched Ca^{2+}-devoid medium. The calculated rate constant for the total EP (i.e., $E_1P + E_2P$) dephosphorylation was 7.8 s^{-1}. The E_1P had an affinity for ADP with an apparent $K_d \simeq 100\ \mu M$. When the EP was formed in the absence of K^+ for 116 ms, no appreciable amount of the ADP-sensitive E_1P was detected. The EP comprised about 80% ADP- and K^+-reactive E_2P and 20% residual EP_r, suggesting a rapid $E_1P \rightarrow E_2P$ transformation. Both the E_2P's formed in the presence and absence of K^+ decomposed with a rate constant of about 19.5 s^{-1} in the presence of 80 mM K^+ and 2 mM ADP, showing an ADP enhancement of the E_2P decomposition. The results demonstrate mechanistic differences in monomeric EP transformation and decomposition between the Triton X-100 purified cardiac SR Ca^{2+}-ATPase and deoxycholate-purified skeletal enzyme [Wang, T. (1986) *J. Biol. Chem. 261*, 6307-6319].

In the course of comparative transient-state kinetic studies of SR,[1] we observed that phosphorylation by ATP of the fast-twitch cat caudofemoralis and tibialis and rabbit back muscle SR gave the total acid-stable EP a greater fraction of the ADP-sensitive E_1P than the slow-twitch cat soleus and dog cardiac SR. The rate of the ADP-enhanced decomposition of the ADP-insensitive E_2P in the presence of K^+ was also

† This work was supported by National Institutes of Health Grant P01 HL22619 (IVA-1).

[1] Abbreviations: SR, sarcoplasmic reticulum; EP, phosphoenzyme intermediate of SR Ca^{2+}-ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

0006-2960/87/0426-8360$01.50/0 © 1987 American Chemical Society