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Origin of the γ polypeptide of the Na^+/K^+-ATPase

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The Na^+/K^+-ATPase purified from lamb kidney contains a γ polypeptide fraction which is a collection of fragments derived from the α and β polypeptides of the enzyme. This fraction has the solubility characteristics of a proteolipid and was isolated either by high performance liquid chromatography (size exclusion chromatography) in 1% sodium dodecyl sulfate or by sequential organic extraction of purified lamb kidney Na^+/K^+-ATPase. Formation of γ polypeptide(s) from detergent solubilized holoenzyme was accelerated by sulfhydryl containing reagents and was unaffected by addition of inhibitors of proteolytic enzymes. Treatment of the holoenzyme with the photoaffinity reagent *N*-(2-nitro-4-azidophenyl)[^{3}H]ouabain ([^{3}H]NAP-ouabain) labeled the α polypeptide and the γ polypeptide fraction but not the β polypeptide. Amino acid sequence analysis of one γ polypeptide preparation revealed homology of one component of this fraction with the *N*-terminus of the β subunit of the Na^+/K^+-ATPase. Amino acid analysis of two preparations of proteolipid showed similar amino acid compositions with a peptide derived from the α subunit. The insolubility and complexity of the γ polypeptide(s)/proteolipid fraction appears to preclude a conclusive sequence analysis of all components of this fraction.

Introduction

The Na^+/K^+-ATPase is composed of two major polypeptides, the 112000 M_r β polypeptide. Several reports [1–5] have indicated the presence of a third polypeptide of molecular weight around 12000 called the γ polypeptide. Labeling of the holo-Na^+/K^+-ATPase with a radioactive photoaffinity derivative of ouabain, either *N*-(2-nitro-4-azidobenzoyl)ouabain (NAB-ouabain) [1], or *N*-(2-nitro-4-azidophenyl)ouabain (NAP-ouabain) [2], followed by solubilization and separation of the subunits by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that a small peptide was specifically labeled. This gave support to the suggestion that the γ polypeptide was a portion of the Na^+/K^+-ATPase and not an exogeneous small peptide co-purified with the Na^+/K^+-ATPase.

Methods

Enzyme preparation. The Na^+/K^+-ATPase enzyme with specific activities of 800–1100 μmol P_i formed per mg protein per h was prepared from the outer medulla of sheep kidneys by published methods [6] and was stored on ice until used. This enzyme contained 1–1.3 μmol phospholipid/mg protein. The enzyme was solubilized for size exclusion chromatography by mixing the Na^+/K^+-

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ATPase suspension (10–20 mg protein/ml) with (final concn.) 2.5% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 100 mM Tris-HCl (pH 6.5) to yield a final Na^+/K^+-ATPase of 1 mg protein/ml. This sample was centrifuged at 100 000 × *g* for 30 min in a Beckman Airfuge. The supernatant was used in subsequent studies.

Size exclusion chromatography. A Gilson High Performance Liquid Chromatograph (HPLC) interfaced to an Apple IIe computer was used with a Beckman TSK-3000 column, an Altex TSK-3000 guard column and a Krytos Variable Wavelength detector. The elution solvent was 1% SDS, 100 mM Tris-HCl (pH 6.5), run at 0.6 ml/min. Usually 100 μg of protein was injected for each analysis. Where indicated fractions were collected at 20-s intervals. For large scale preparation, samples up to 2 mg protein per injection were used and adequate resolution of peaks was maintained. The proteolipid fraction was collected between 13.4 and 14.9 min after injection.

The SDS and phospholipid content of the proteolipid fraction from the HPLC runs could be greatly reduced by dialysis. The sample was placed in a 3500 molecular weight cut off 'Spectopore' dialysis bag and was initially dialyzed versus water. Over a 4-h period the composition of the dialyzate was changed until it contained 50% methanol. The dialyzate could then be changed to 100% methanol without the γ polypeptide precipitating, then the composition of the dialyzate was changed until a ratio of 2 : 1 chloroform/methanol was attained. After 12 h in chloroform/methanol the sample was dialyzed back into methanol. This dialysis reduced the SDS content from 10 mg/ml to 0.04 mg/ml and the phospholipid phosphorus content from 9 μmol/mg protein to approximately 0.5 μmol/mg protein. All water had to be excluded from contact with the sample or the proteolipid would irreversibly precipitate from solution. Care was needed as the dialysis bags became brittle in organic solvent and broke easily. Returning the bags to an aqueous media permitted a return of their normal physical characteristics.

Action of proteolytic enzyme inhibitors. For examination of the action of proteolytic enzyme inhibitors, the Na^+/K^+-ATPase was solubilized without the reducing agent and divided into aliquots. A sample without 2-mercaptoethanol was used as a control. Other aliquots were treated with: (1) 1% 2-mercaptoethanol; (2) boiling water bath for 20 min, cooling and then the reducing agent was added; (3) 25 mM EDTA and 1% 2-mercaptoethanol; (4) 2 mM phenylmethylsulfonyl fluoride in ethanol added, then the reducing agent was added; (5) pepstatin A at a 1 : 100 weight ratio of inhibitor to enzyme and the reducing agent added; (6) soybean trypsin inhibitor added at a 1 : 100 weight ratio, then reducing agent added; (7) 2 mM *N*-ethylmaleimide or iodoacetamide solutions were added; the reaction was run for 1 h at room temperature, then 2-mercaptoethanol was added. Analysis of each sample on the TSK-3000 column was run immediately after the indicated treatment. This served as data for day zero. Samples were kept at room temperature and analyzed for change in elution profile by size exclusion chromatography at 2-day intervals for the following fourteen days.

Synthesis of NAP-ouabain. Ouabain, $NaIO_4$ and $NaCNBH_4$ were purchased from Aldrich Chemical Co; [^{3}H]ouabain purchased from New England Nuclear had a specific activity of 20 Ci/mmol. *N*-(2-aminoethyl)-4-azido-2-nitroaniline was purchased from Molecular probes, Eugene, OR. NAP-ouabain or [^{3}H]NAP-ouabain were synthesized by a modification of published methods [1,2]. Ouabain was oxidized in water with 10% molar excess of sodium periodate at room temperature in the dark for 2 h. The periodate anion was removed by elution with water on a Dowex 1X8 (200–400 mesh) column, in the chloride form. The oxidized ouabain column effluent was taken to dryness under vacuum and was redissolved in methanol. A molar equivalence of *N*-(2-aminoethyl)-4-azido-2-nitroaniline was added followed by a 20% molar excess of $NaCNBH_4$. The reaction was run for 16–24 h, at room temperature in the dark. The dark orange-red solution was centrifuged for 3 min. The product was isolated from the supernatant using a C18 reverse phase column (Vydac, 0.46 × 25 cm) with a H_2O-methanol gradient from 30% methanol in H_2O to 100% methanol at a flow rate of 1 ml/min. Using this system the following elution times were observed: original ouabain, 8.2–9.0 min; oxidized ouabain, 9.5–11 min; azidonitroaniline, 13.5–14.2 min; and the desired product, 16.6–18.0 min. In

addition to ouabain only the peak between 16.6–18 min specifically bound to the Na^+/K^+-ATPase and inhibited enzyme activity.

A molar absorption coefficient of 5000 $M^{-1} \cdot cm^{-1}$ at 460 nm for the phenyl azide [2] was used to determine the stoichiometry between ouabain and the phenyl azide in the product. Ouabain concentration was calculated by using the specific radioactivity of the starting ouabain. Ratios of between 0.96 to 1.04 mol of ouabain per mol of phenyl azide were found. The presence of an active azido group was established by comparing the absorption spectra between 300–530 nm of the original sample kept in the dark and a photolyzed aliquot [1]. The [^{3}H]NAP-ouabain was kept in dried aliquots in the dark at 0 °C. Yields of 8–13% of the product to the starting radioactive ouabain were routinely obtained. The final product contained 70000–110000 dpm/nmol.

The Na^+/K^+-ATPase was labeled with the [^{3}H]NAP-ouabain following the procedure for ouabain binding [7]. The enzyme was preincubated either in a medium containing 100 mM NaCl, 3 mM $MgCl_2$, 3 mM Na_2ATP and 40 mM Tris-HCl (pH 7.4) or in a medium containing 150 mM Tris-HCl, 5 mM EDTA containing 10 μM unlabelled ouabain at 37 °C in the dark for 15 min. Then [^{3}H]NAP-ouabain was added in 10–100-fold excess over potential binding sites (2 nmol/mg protein) for an additional 20 min at 37 °C in the dark. The samples were centrifuged at 35000 rpm in a Spinco 40 rotor and were washed two additional times in fresh binding buffer. The final pellets were resuspended in fresh original buffer placed in quartz cuvettes, exposed to a 'Pen Ray Ultraviolet' light source 2 cm away for 2–10 min, and were centrifuged at 35000 rpm in a Spinco 40 rotor for 20 min. Aliquots were counted for radioactivity in scintillation cocktail [7] and a portion was analyzed for protein.

Organic extraction of proteolipid. Aliquots of the holo Na^+/K^+-ATPase or [^{3}H]NAP-ouabain labeled enzyme were extracted twice with cold methanol (90% methanol in the final extract), three times with cold chloroform/methanol (2 : 1, v/v), and then at room temperature with chloroform/methanol containing 10 mM HCl [1]. The acidified organic solvent was reduced in volume by evaporation under nitrogen. The residual pellet was insoluble after organic extractions therefore it was hydrolyzed in 2 M NaOH before radioactivity was measured.

In a second method [3] between 2 and 4 mg of Na^+/K^+-ATPase in aqueous medium was extracted with 10 volumes of chloroform/methanol containing 10 mM HCl. The proteolipid was precipitated from the organic phase by the addition of 5 vols. of diethyl ether and the sample was maintained at −20 °C. The proteolipid was collected by centrifugation. The pellet was resuspended in acidified chloroform/methanol (2 : 1) which solubilized most of the sample.

SDS-urea polyacrylamide gel electrophoresis. SDS-urea polyacrylamide gel analysis was performed as previously described [8] with the modification that the resolving gel was 15% polyacrylamide containing 3 M urea.

Amino acid sequence analysis. The gas phase amino acid sequence analyses [16] were performed by the Protein Chemistry Core facility of the Department of Pharmacology and Cell Biophysics at the University of Cincinnati supervised by Dr. T.L. Kirley.

Assays. Protein determination of the holo-Na^+/K^+-ATPase was run using bovine serum albumin as a standard [9]. For the proteolipid fractions a modified procedure was followed [10]. Total lipid extraction of the samples was performed [11] and the extracted lipids were analyzed for phospholipid phosphorus (PLP) [12]. SDS was measured by an established method [13].

Results

Isolation of γ polypeptide by size exclusion chromatography

After membrane disruption by detergent the polypeptide subunits of purified Na^+/K^+-ATPase were separated and isolated by size exclusion chromatography. Using HPLC with a TSK-3000 column and 1% SDS-100 mM Tris HCl (pH 6.5) as solvent, fractions containing the 112000 M_r α subunit, the 35000 M_r β subunit and a smaller 11000 M_r γ polypeptide(s) were isolated. The γ polypeptide fraction eluted from the column with phospholipids (Fig. 1). This makes the presence of the 11 kDa polypeptide easily overlooked by this

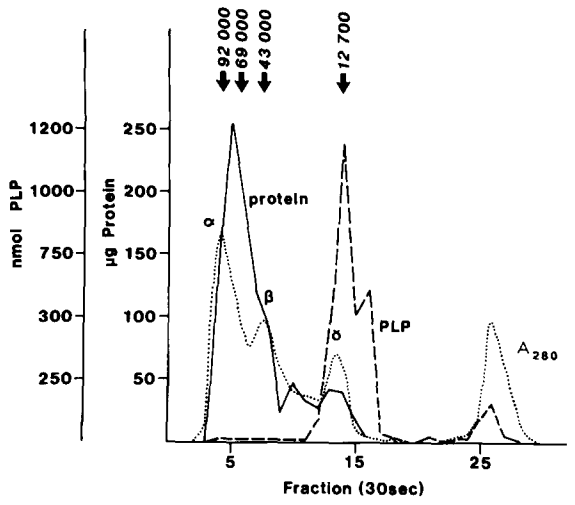

Fig. 1. Isolation of Na^+/K^+-ATPase components by HPLC on size exclusion column. Na^+/K^+-ATPase was solubilized in (final concn.) 2.5% SDS containing 1% 2-mercaptoethanol and 100 mM Tris-HCl (pH 6.5). 2 mg of preparation were injected into a 0.75×30 cm TSK-3000 column with a running solvent of 1% SDS, 100 mM Tris-HCl (pH 6.5). A_{280nm} (·····), protein content [10] (———) and phospholipid phosphorus (— — —) were monitored on fractions collected at 30-s intervals. Positions of elution of standard proteins are indicated. α, β and γ polypeptides were resolved by this procedure.

technique. By monitoring the absorbance of the effluent at 280 nm, the γ polypeptide comprised approximately 14% of the integrated area of a freshly solubilized sample but as shown in Fig. 1 only 7% of the total protein content as measured with a modified protein analysis [9]. The difference in results between the techniques was due to light scatter caused by phospholipid micelles in the detergent. When the holo-enzyme was chromatographed, over 80% of the phospholipid eluted with the retention time of the γ polypeptide(s) (Fig. 1). However, the elution profile of a lipid extract of the Na^+/K^+-ATPase showed that only 40–50% of the lipids eluted with the same retention time of the γ polypeptide(s). The remainder of the lipids had longer retention times, i.e. smaller apparent molecular weight. The γ polypeptide(s) was present in every lamb kidney Na^+/K^+-ATPase preparation that we examined. Its proportion of the total protein varied slightly from preparation to preparation.

Effects of 2-mercaptoethanol and proteolytic enzyme inhibitors

The presence of the reducing agent 2-mercaptoethanol in the solubilization solution caused a slight increase in the proportion of γ polypeptide(s) in the elution profile on the TSK-3000 column immediately after solubilization. The effect of this reducing agent on the amount of γ polypeptide(s) became pronounced when the solubilized sample was kept at room temperature for extended periods of time (Fig. 2). After thirteen days the proportion of γ polypeptide(s) had increased from 15% to as high as 33% of the area of the 280 nm absorbing materials in the presence

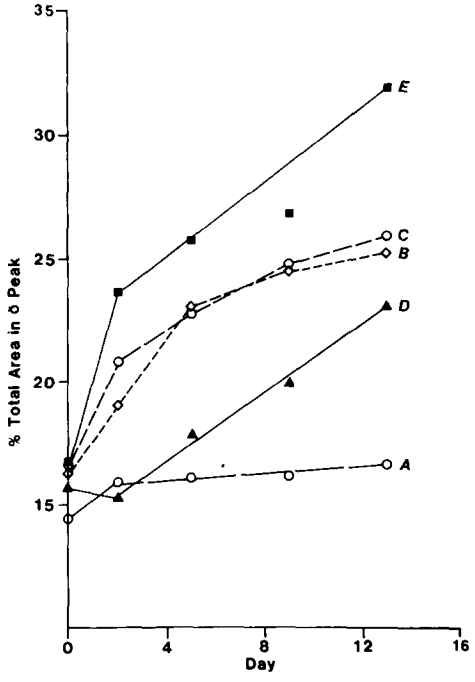

Fig. 2. Effect of 2-mercaptoethanol and proteinase inhibitors on formation of γ polypeptide. Na^+/K^+-ATPase was solubilized in 2.5% SDS, treated as indicated below and allowed to stand in sealed tubes at room temperature for up to 13 days. Aliquots were analyzed by size exclusion HPLC at the times indicated using conditions identical to Fig. 1. The proportion of the total integrated area, measured at 280 nm, found in the γ polypeptide is shown. A, ○------○, control sample without 2-mercaptoethanol. B, ◇------◇, control with 1% 2-mercaptoethanol. C, ○------○, sample heated at 100 °C then 2-mercaptoethanol added. D, ▲———▲, sample with 25 mM EDTA and 2-mercaptoethanol. E, ■———■, sample with 2 mM iodoacetamide and 2-mercaptoethanol.

of 2-mercaptoethanol (Fig. 2B) while the control sample without the reducing agent increased from 14 to 15.5% (Fig. 2A). While the proportion of the γ polypeptide(s) peak was increasing the proportion of the α and β peaks was decreasing (data not shown).

Effects of reagents or conditions that should inhibit proteolytic enzyme activity, especially sulfhydryl requiring proteinases were also examined. The Na^+/K^+-ATPase was solubilized in 2.5% SDS, divided into aliquots, treated to inhibit proteinase activity and then 2-mercaptoethanol was added. Conditions used included 25 mM EDTA (Fig. 2D), heating to 100°C for 20 min (Fig. 2C) and iodoacetamide (Fig. 2E, to block free SH on either the Na^+/K^+-ATPase or proteinases). The elution profile of the Na^+/K^+-ATPase on the TSK-3000 column was examined periodically for thirteen days after solubilization. None of the conditions used including the use of *p*-methylsulfonylfluoride, soybean trypsin inhibitor, or pepstatin A (data not shown) seemed to affect the formation of the polypeptide at the short time intervals after addition of 2-mercaptoethanol (Fig. 2), EDTA seemed most effective and appeared initially to retard the γ polypeptide(s) formation (Fig. 2D) but after 14 days the EDTA sample had the same proportion of γ polypeptide(s) as the control sample with the reducing agent. The reaction of SH groups of the Na^+/K^+-ATPase preparation with either *N*-ethylmaleimide (data not shown) or iodoacetamide (Fig. 2E) caused an increase in the proportion of γ polypeptide(s) over the control sample containing 2-mercaptoethanol (Fig. 2B). This could be due to the decreased number of free protein SH groups, lowering the possibility of interpeptide disulfide exchange and favoring formation of intrapeptide disulfide bonds or breaking of disulfide bonds.

The α polypeptide was isolated from a TSK-3000 analysis in 1% SDS, 100 mM Tris-HCl (pH 6.5). One aliquot was made 1% in 2-mercaptoethanol and the other did not contain reducing agent. Both aliquots degraded into 11 kDa and smaller polypeptides. The rate of breakdown was much greater with reducing agent than in its absence (data not shown). These data suggest that the increase in proportion of the 11 kDa γ polypeptide(s) is not due to an intrinsic proteinase in the Na^+/K^+-ATPase preparation but does not exclude this possibility.

Comparison of γ polypeptide(s) and proteolipid materials

Different techniques have been used to obtain a proteolipid fraction including size exclusion chromatography, SDS-PAGE, and organic extraction of the Na^+/K^+-ATPase. To determine if the same material was isolated using these different techniques the proteolipid from the Na^+/K^+-ATPase preparation was treated by sequential organic extraction [1]. The proteolipid solvent was changed by dialysis from organic solvent to the aqueous SDS solubilization solvent used for the holo-Na^+/K^+-ATPase. This proteolipid eluted from the TKS-3000 column in the same position as the γ polypeptide(s) of the holoenzyme solubilized in SDS (data not shown). A mixture of the proteolipid and the γ polypeptide(s) eluted as a single peak when rechromatographed on the TKS-3000 column which demonstrates that they have similar apparent molecular weights.

SDS-PAGE analysis

The 11 kDa polypeptide isolated by size exclusion chromatography was resolved as a diffuse band by gel electrophoresis on a 15% SDS-urea polyacrylamide gel (Fig. 3). It migrated slightly behind insulin (6 kDa). The γ fraction from TSK-3000 chromatography or the proteolipid isolated by organic extraction migrated to the same position (data not shown) and indicated that they have similar molecular weights.

On 5–10% polyacrylamide gels the 11 kDa polypeptide(s) was very difficult to locate as it migrated just behind the dye front with most of the phospholipids of the preparation. Apparently in 5–10% acrylamide gels this material was poorly fixed with 10% acetic acid-isopropanol as the initial faint Coomassie blue band could not be located 16 h later.

[^{3}H]NAP-ouabain binding

[^{3}H]NAP-ouabain was synthesized, bound and photolyzed to the Na^+/K^+-ATPase as described in the Methods. In the presence of NaCl, $MgCl_2$, ATP and buffer, the enzyme bound 3.6 nanomol [^{3}H]NAP-ouabain/mg protein and without

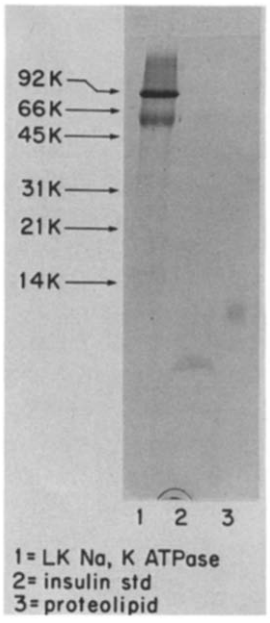

Fig. 3. Identification of peptides by SDS-urea polyacrylamide gel electrophoresis. Na^+/K^+-ATPase was solubilized and separated into components on a 15% polyacrylamide resolving gel, as described in Methods (lane 1). Insulin was run in lane 2 and the proteolipid fraction in lane 3. The positions of molecular weight markers are shown on the left margin. The gel was stained with Coomassie blue dye and destained in 10% acetic acid.

ligands 0.32 nanomol bound/mg protein. After binding and photolyzing [^{3}H]NAP-ouabain to the enzyme in the presence of ligands, the enzyme was solubilized in SDS-2-mercaptoethanol-Tris buffer and chromatographed on the TKS-3000 column. The α polypeptide bound 2.5–3.4% of the recovered [^{3}H]NAP-ouabain while the γ polypeptide fraction contained 88–93% of the recovered tritium. The majority of the [^{3}H]NAP-ouabain in the γ polypeptide fraction was associated with phospholipids (Harris and Stahl, unpublished data). These phospholipids were separated from the γ polypeptide(s) by either dialysis or by chromatography on a LH-60 Sephadex column (Methods). After removal of the phospholipids and the [^{3}H]NAP-ouabain associated with the phospholipids, protein in the γ polypeptide fraction bound between 1.8 to 2.2% of the recovered [^{3}H]NAP-ouabain. This proportion was slightly less than the amount bound to the α polypeptide.

Alternatively, the [^{3}H]NAP-ouabain labeled-Na^+/K^+-ATPase was extracted with organic solvent to yield the proteolipid fraction. The proteolipid fraction retained 1.4–3.2% of the initial [^{3}H]NAP-ouabain specifically bound to the enzyme. These data from size exclusion chromatography, SDS-PAGE and [^{3}H]NAP-ouabain binding suggest that the γ peak and the extracted proteolipid are the same material.

Removal of phospholipids from proteolipid fraction

After organic extraction the proteolipid had 1–2 μmol of associated phospholipid phosphorus per mg of protein. The amount of phospholipid bound to the proteolipid had a major influence of the solubility of the preparation. By exhaustive dialysis versus chloroform/methanol (2:1) the phospholipid content could be reduced to 0.3–0.5 μmol phospholipid phosphorus/mg protein. The phospholipid content of the proteolipid fraction could be further reduced by chromatography on a

TABLE I

AMINO ACID COMPOSITION OF Na^+/K^+-ATPase PROTEOLIPID FRACTION

Amino acid composition of the Na^+/K^+-ATPase proteolipid preparation I (column A), proteolipid preparation II (column B) and tryptic digest of α-polypeptide [16] (column C) were measured as described in Methods.

Amino acid	Composition (residues per 100 residues)		
	A	B	C
Asx	8.4	8.5	7.6
Glx	11.1	10.9	10.4
Ser	7.1	7.1	6.8
Gly	9.2	8.1	9.3
His	1.6	2.1	2.0
Arg	4.8	5.5	3.2
Thr	5.5	5.8	6.0
Ala	8.1	8.6	9.7
Pro	6.4	5.2	3.6
Tyr	3.1	3.4	3.4
Val	6.7	6.1	6.3
Met	2.1	2.3	2.4
Cys	–	–	1.1
Ile	5.5	4.9	6.8
Leu	10.5	11.5	11.0
Phe	4.7	5.1	5.7
Lys	5.3	4.8	4.8

TABLE II

AMINO ACID SEQUENCE OF Na^+/K^+-ATPase PROTEOLIPID FRACTION

Amino acid sequence of Na^+/K^+-ATPase proteolipid fractions I and II and N-terminal of β-subunit (Ref. 15) of Na^+/K^+-ATPase as determined in Methods.

A. Proteolipid I	Ala-Arg-Gly-xxx-Ala-Lys-Glu-Glu-Gly
B. Proteolipid II	Ala-xxx-Pro-Phe-(Lys)Tyr-Tyr-(Ile)Phe
C. N-terminal β	Ala-Arg-Gly-Lys-Ala-Lys-Glu-Glu-Gly

LH-60 Sephadex column using chloroform/methanol (2:1), 0.1% trifluoroacetic acid as the solvent. This technique reduced the phospholipid content in the fraction to less than 0.1 μmol phospholipid phosphorus per mg protein. When the phospholipid content was in this range, the proteolipid was subjected to gas phase amino acid sequence analysis.

Amino acid sequence analysis

Two proteolipid fractions prepared from the same stock of purified lamb kidney Na^+/K^+-ATPase were analyzed for amino acid sequence. The only difference between the two proteolipid fractions was the date of isolation. The amino acid sequence analysis of the first proteolipid sample gave the sequence shown in Table II, line A and the total amino acid composition shown in Table I, column A. This sequence is homologous to the N-terminus of the β-polypeptides, (Table II, line C) [15] and would appear to be derived from it. However, the total recovery of materials from the analysis was only 29% of the material applied to the filter for sequencing. The second proteolipid sample analyzed by this technique produced the sequence shown in Table II, line B and the amino acid composition shown in Table I, column B. This sequence was not found in either the α or β subunits sequences of the Na^+/K^+-ATPase. With this sample less than 10% of the initial materials could be found after eight cycles of analysis and only 49% of the applied sample could be found anywhere in the system including on the filter and waste solvents.

The total amino acid composition of the two proteolipid samples, (Table I) are almost identical. We suspect that the proteolipid II was a mixture of peptides extracted by this technique. This indicates that the solubility characteristics of the proteolipid fraction limit extensive sequence analysis by this method. Column C in Table I presents the amino acid composition of a collection of peptides obtained by trypsin hydrolysis of the α peptide of the Na^+/K^+-ATPase [16]. These data are almost identical to the proteolipid's amino acid composition, Table I, columns A and B. However, the composition of the proteolipid fraction is probably complex and has resisted attempts to separate individual polypeptides by reverse phase HPLC using a variety of columns and solvent systems (data not shown).

Discussion

The presence of a small molecular weight γ polypeptide or proteolipid in Na^+/K^+-ATPase preparations has been reported by a number of laboratories [1–5]. The NAP-ouabain [2] and NAB-ouabain [1], photoaffinity derivatives labeled a proteolipid in the Na^+/K^+-ATPase preparation as well as the α subunit suggesting that the proteolipid might be an integral part of the Na^+/K^+-ATPase. A total of 0.9 mol of proteolipid peptides per α subunit of the Na^+/K^+-ATPase has been reported [5]. A stoichiometry of 2 mol of proteolipid per $\alpha_2\beta_2$ configuration of the shark rectal gland Na^+/K^+-ATPase was reported [17]. Using size exclusion HPLC we found that the proportion of proteolipid peptides to α and β subunits could be increased by incubation of the holoenzyme with 2-mercaptoethanol in detergent.

Several possibilities could account for the presence of the proteolipid polypeptide in the Na^+/K^+-ATPase preparation: (1) This polypeptide could be a contaminant that was copurified with the Na^+/K^+-ATPase. (2) It could be a separate integral polypeptide of the Na^+/K^+-ATPase complex of unknown function. (3) It could be a peptide breakdown product of either the α or β subunits. The first and second possibilities seem unlikely since the proportion of γ in relation to the total protein varied with time and the presence of a reducing agent.

The third possibility seemed more likely and a small proteolytic enzyme could have been co-

purified with the Na^+/K^+-ATPase. The presence of such an enzyme would be more evident from its proteolytic action than its physical properties. A series of treatments was used to reduce or eliminate protease activity. None of these treatments substantially inhibited the formation of γ polypeptide over the control without inhibitor. The treatment of the Na^+/K^+-ATPase with either *N*-ethylmaleimide or iodoacetamide actually accelerated the formation of the γ fraction over that of the controls (Fig. 3). We have no definitive evidence to support the possibility of an exogenous proteolytic enzyme being co-purified with the lamb kidney Na^+/K^+-ATPase.

The breakdown of the α and β polypeptides could be catalyzed by breaking critical disulfide bridges. The Na^+/K^+-ATPase in an $\alpha_2\beta_2$ structure from lamb kidney contains 60 cysteine/cystine residues; 23 residues in the α subunit and 7 residues per β subunit [18,15]. Up to 36 mol of free sulfhydryl per mol of $\alpha_2\beta_2$ structure have been reported [19]. This allows 26–32 mol of 1/2 cystine residues that might be involved in disulfide bridges of an intra- or interpeptide nature. If the γ proteolipid were formed as a result of disulfide exchange with the exogenous 2-mercaptoethanol or intrapeptide exchange it must contain free SH groups or 1/2 cystine residues. One Na^+/K^+-ATPase proteolipid characterized [5] contained 2.28 (peptide I) or 2.14 (peptide II) residues of converted cysteic acid per 100 residues. Another report [19] found 0.8 mol of SH per mol of ATPase-proteolipid isolated from shark rectal gland. We have measured 1.8–2 mol of free SH per mol of 11 kDa polypeptide in the γ polypeptide fraction collected from the size exclusion column. The presence of cysteine/cystine residues in the proteolipid suggests to us that disulfide exchange could be a contributing factor in formation of the γ polypeptide fraction. Perhaps reduction of a critical disulfide may make a previously buried segment of the polypeptide susceptible to cleavage.

Information obtained by size exclusion chromatography, SDS-polyacrylamide gel electrophoresis and [^{3}H]NAP-ouabain binding show that the γ polypeptide(s) obtained from HLPC chromatography is the same material as isolated by organic extraction of the Na^+/K^+-ATPase the proteolipid fraction. This is likely the same material isolated or reported previously by others [1–5].

There is little doubt that at least one component in the proteolipid fraction is derived from the β subunit since amino acid sequence homology was found (Table II). The presence of peptides derived from the α subunit is more difficult to establish. The amino acid composition of the γ polypeptide(s) is very similar to the composition of a tryptic digest of the α polypeptide. Comparing the γ proteolipid to the tryptic digest of the α subunit for relatedness of two proteins [20] the sum of squared difference produced a value of 15. Values over 50 are an indication of unrelated proteins while those under 50 are considered related. This would be expected if a major proportion of polypeptides present in the γ proteolipid fraction was derived from the α polypeptide. The labeling of the γ polypeptide by the ouabain photoaffinity derivative could also be explained if the γ polypeptide was a portion of that subunit of the Na^+/K^+-ATPase. The idea that the γ polypeptide fraction is derived from α and β subunits is also supported by the finding that treatment of holoenzyme in detergent and 2-mercaptoethanol decreased the amount of the α and β polypeptide peaks and increased the proportion of γ polypeptide peak on size exclusion HPLC (Fig. 2B). We cannot completely exclude the presence of a polypeptide in the γ fraction which is not derived from α and/or β subunits, since not all polypeptides were recovered for amino acid sequence analysis.

From this study we conclude that the γ polypeptide or proteolipid fraction contains breakdown products of the β subunit and possibly of the α subunit of the Na^+/K^+-ATPase. The relative insolubility of the fraction suggests that it also contains limit polypeptides, probably containing membrane spanning regions of the Na^+/K^+-ATPase. These would be expected to be highly hydrophobic and intractable to sequence analysis. Formation of the proteolipid fraction may be catalyzed by breaking critical disulfide bonds in the holo-enzyme. The Na^+/K^+-ATPase may possess auto-proteolytic activity under these conditions or there could be a minor contaminant of a proteolytic enzyme present in our kidney prepara-

tions of the Na^+/K^+-ATPase. Disulfide bonds may play a more significant role in maintaining the protein's tertiary structure in membrane proteins than in soluble proteins. This would be especially true when the membrane proteins are removed from the surrounding lipid bilayer such as in SDS 'solubilized' proteins.

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