Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 64-70

IDENTIFICATION OF A DISULFIDE BETWEEN CYSTEINE 214 AND CYSTEINE 277 IN THE β SUBUNIT OF NATIVE (Na^++K^+)ATPase

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Received May 30, 1989

SUMMARY. Two peptides, produced during tryptic digestion and thermolytic digestion, repectively, and containing the same intact disulfide from the β polypeptide of (Na^++K^+)ATPase from Torpedo californica, were isolated and unambiguously identified. The disulfide is between Cysteine 214 and Cysteine 277. © 1989 Academic Press, Inc.

Sodium and potassium ion-activated adenosine triphosphatase [(Na^++K^+) ATPase] is an integral membrane protein present in most eukaryotic cells. It is responsible for maintaining an electrochemical gradient across the plasma membranes of these cells by catalyzing the transport of three sodium ions out of the cell and two potassium ions into the cell coupled to concomitant hydrolysis of MgATP. It is composed of two polypeptides, an α polypeptide with a length of 1016 amino acids, which contains the binding sites for ATP and cardiac glycoside (1, 2), and a β polypeptide with a length of 305 amino acids, which is a sialoglycoprotein of unknown function (3). Sequences for the α polypeptide from sheep, pig, and T. californica, and the β polypeptide from pig, dog, rat, man, sheep, and T. californica, have been determined (4-10). Canine, porcine, human, and ovine β polypeptides from (Na^++K^+) ATPase are homologous to each other (90% frequency of identity), and they are also homologous (60% frequency of identity) to β polypeptide from T. californica (7). All have a hydrophobic segment near their amino terminus approximately 20 amino acids in length that is thought to anchor the polypeptide in the membrane. All β polypeptides of (Na^++K^+) ATPase sequenced thus far contain seven conserved cysteines and three conserved, potential sites for N-linked glycosylation. A disulfide between Cysteine 158 and Cysteine 174 of canine renal (Na^++K^+) ATPase β polypeptide has been identified previously (11,12).

The electric organ of T. californica contains large concentrations of acetylcholine receptor, (Na^++K^+)ATPase, and acetylcholine esterase (13). During the course of my purifications of cystine-containing peptides (14) from proteolytic digests of the polypeptides of acetylcholine receptor from T. californica, two peptides, produced during tryptic digestion and

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0006-291X/89 $1.50
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thermolytic digestion, respectively, and containing the same intact cystine from the β polypeptide of (Na^++K^+)ATPase, were isolated and unambiguously identified.

MATERIALS AND METHODS Sodium dodecyl sulfate ($NaDodSO_4$) was recrystallized from 95% ethanol. Iodoacetamide and o-phthalaldehyde were recrystallized from n-heptane. Trifluoroacetic acid, phenylisothiocyanate, triethylamine, and 88% formic acid were redistilled before use. n-Propanol and isopropanol of a purity suitable for high pressure liquid chromatography were from Burdick and Jackson; thermolysin, from Sigma Chemical Co.; Bio-gel A5m from Biorad; and trypsin, treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, from Worthington Biochemical Corp.

Purification of acetylcholine receptor. Acetylcholine receptor was purified in the presence of N-ethylmaleimide by the method of Elliot et al. (15) as modified by Kellaris and Ware (14).

High pressure liquid chromatography (HPLC). Samples were injected onto a Vydac reverse-phase C_4 column (0.46 x 25 cm) or a Brownlee Labs reverse-phase C_8 column (0.46 x 25 cm). The effluent from the detector was directed into an electrochemical cell designed for the detection of disulfides with the following components from Bioanalytical Systems Inc.: two model LC-4B amperometric controllers, a dual gold/mercury electrode with a collection port, and a model RE3 reference electrode. To detect cystine with the electrochemical cell, a reduction (-1.00 V) was carried out at the first electrode and an oxidation (+0.156 V) was carried out at the second electrode. Current at the second electrode was monitored at 20 or 50 nanoamperes full scale.

General methods. This report describes two unexpected results encountered during a systematic examination of the cystines of acetylcholine receptor (14). The isolation of the denatured polypeptides, gel electrophoresis, thermolytic digestion, tryptic digestion, molecular exclusion chromatography on Fractogel TSK HW40s (Supelco Co.), HPLC analysis of phenylthiocarbamyl (PTC) amino acids, analysis for glucosamine, amino acid analysis, and amino acid sequencing were performed exactly as described in the earlier publication reporting the other results (14).

RESULTS

A sample (95 mg protein) of membranes, enriched in acetylcholine receptor, was alkylated at a final concentration of 5 mM N-ethylmaleimide while $NaDodSO_4$ was added at a fourfold excess by weight over protein. After 30 min at 37°C, the sample was divided into two parts. Each part was submitted to chromatography, separately, on a column of Bio-gel A5m (2.6 x 90 cm) equilibrated in 0.1% $NaDodSO_4$, 40 mM Tris sulfate, pH 8.0 (3), and the polypeptides were eluted at a flow rate of 7.2 mL h^{-1}. The distribution of the α, β, γ, δ, and δ_2 polypeptides of acetylcholine receptor over the elution profile was determined electrophoretically. Fractions containing various mixtures of polypeptides were pooled from both runs and concentrated by lyophilization. The dodecyl sulfate was removed from the polypeptides exactly as described by Nicholas (16).

Cystine-containing Peptide (β183-β217)-(β275-β278) of (Na^++K^+)ATPase. The pool from the column of Bio-gel A5m containing the α, β, and γ polypeptides of acetylcholine receptor (distribution coefficient of 0.50) was digested with 10 μg trypsin (mg protein)$^{-1}$ at 37°C. A second, equal addition of trypsin was made after six hours, and the digestion was allowed to continue overnight. The extent of the digestion, determined by treatment of a portion with carboxypeptidase B, was essentially complete, and digestion was terminated by lyophilization. The peptides (29 mg of protein as determined by amino acid analysis) were dissolved in 60% formic acid and submitted to molecular-exclusion chromatography on a column of Fractogel TSK HW40s (0.9 x 50 cm) equilibrated in 95% ethanol:88% formic acid::4:1 (v/v). The peptides were eluted at a flow rate of 4.2 mL h-1, and fractions of 0.7 mL were collected.

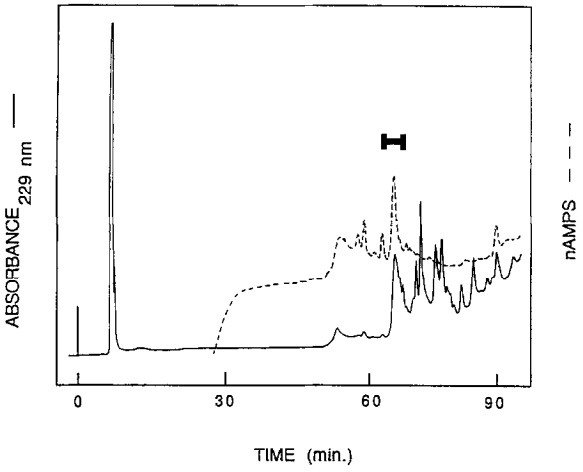

FIGURE 1. Purification of peptide (β183-β217)-(β275-β278) of $(Na^+ + K^+)$ATPase by HPLC on C_4 reverse-phase chromatographic medium. Fraction 17 from the molecular exclusion chromatogram was dissolved in 100 µL of 88% formic acid and injected onto the Vydac C_4 column equilibrated in 0.1M sodium chloroacetate, pH 5.0. The sample was eluted with a linear gradient between Solvent A (0.1M sodium chloroacetate, pH 5.0) and Solvent B (n-propanol) as follows: 0-25 min, 0% B, 0.7 mL min^{-1}; and 25-85 min, 0-60% B, 0.7 mL min^{-1}. Absorbance at 280 nm was monitored at 0.2 absorbance unit full scale and electrochemical detection was performed at a setting of 20 nanoamperes full scale.

Fractions from this chromatogram containing cystine (Fractions 13 through 17; distribution coefficients 0.05 to 0.17) were submitted one at a time to HPLC on the C_4 reverse-phase column developed with a gradient between 0.1M sodium chloroacetate, pH 5.0 and n-propanol (Figure 1). Of the several peaks of electrochemically active peptide detected on each chromatogram, the same one, eluting at 45% n-propanol , was gathered from each chromatographic separation, and all of these were pooled and evaporated. The complete pool was dissolved in 12% formic acid and injected onto the Vydac C_4 column equilibrated in 95% Solvent A (0.1% trifluoroacetic acid) and 5% Solvent B (0.1% trifluoroacetic acid, 60% isopropanol). The sample was eluted with a linear gradient between Solvent A and Solvent B as follows: 0-28 min, 5% B, 0.7 mL min^{-1}; and 29-89 min, 5-60% B, 0.7 mL min^{-1}. Only one peak of absorbance at 229 nm, eluting at 66 min, was electrochemically active. This peak was collected, and the pool was evaporated. It was dissolved in 12% formic acid and injected onto the Brownlee Labs C_8 column equilibrated in 95% Solvent A (0.1% trifluoroacetic acid) and 5% Solvent B (0.1% trifluoroacetic acid, 60% isopropanol). The sample was eluted with a linear gradient between Solvent A and Solvent B as follows: 0-25 min, 5% B, 0.7 mL min^{-1}; and 25-85 min, 5-100% B, 0.7 mL min^{-1}. The single peak, eluting at 63 min on this chromatogram, with both absorbance at 229 nm and electrochemical activity was collected and submitted to amino acid sequencing (Figure 2A), amino acid analysis, and analysis for glucosamine.

The peptide was identified as the tryptic peptide β183-β217 connected through an intact cystine to tryptic peptide β275-β278 (Figure 2B). With the exception of Asx and Ser, the amounts of all of the amino acids expected upon amino acid analysis (Thr, Glx, Gly, Ala, Val,

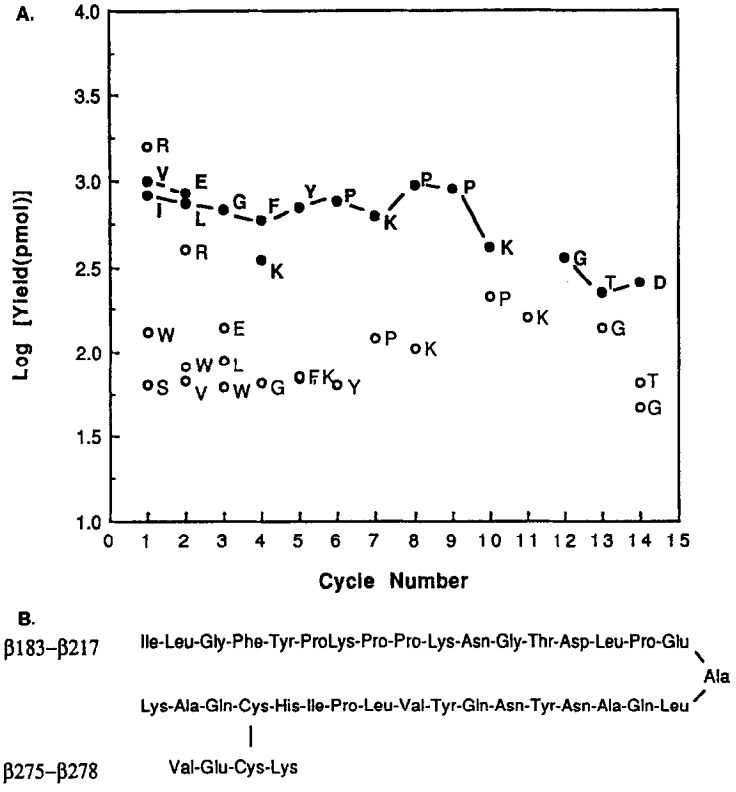

FIGURE 2. Sequence analysis of peptide (β183–β217)–(β275–β278). (A) The logarithms of the yields in picomoles of every phenylthiohydantoin of an amino acid observed on a particular cycle of the automated sequencing are plotted. The amino acids assigned as those from the designated peptide and those expected at a particular cycle are in bold face. (B) Amino acid sequence of the cystine-containing peptide, (β183–β217)–(β275–β278). The sequence presented is from the complete amino acid sequence of the β polypeptide of $(Na^+ + K^+)$ATPase from T. californica (10) and the amino terminus and carboxy terminus were assigned based on the observed sequence and the specificity of the trypsin used to produce the peptide. Cystine is assumed based on the observed amino acid composition.

Ile, Leu, Tyr, Phe, His, and Lys) were within 20% of the stoichiometries in which they appear in this peptide, and those amino acids not expected were present at background levels (<0.1 nmol for every nmol of peptide). There were 0.6 nmol of intact cystine observed upon the chromatogram from the amino acid analyzer for every nmol of the peptide. Although glucosamine was identified in the hydrolysate submitted to phenylthiocarbamylation, it was not quantified.

Final yield of the peptide could not be estimated because the amount of β polypeptide of $(Na^+ + K^+)$ATPase in the initial pools of polypeptide from the column of Bio-gel A5m was unknown. Because 6 nmol of pure peptide was found in the final chromatogram, however, it appears that the β polypeptide of $(Na^+ + K^+)$ATPase was present in fairly high concentrations in the purified membrane preparations and that yield of the purified peptide from the β polypeptide was reasonably high. This follows from the fact that the yields of cystine-containing peptides of acetylcholine receptor from this same preparation were between 2 and 6 nmoles for each peptide isolated.

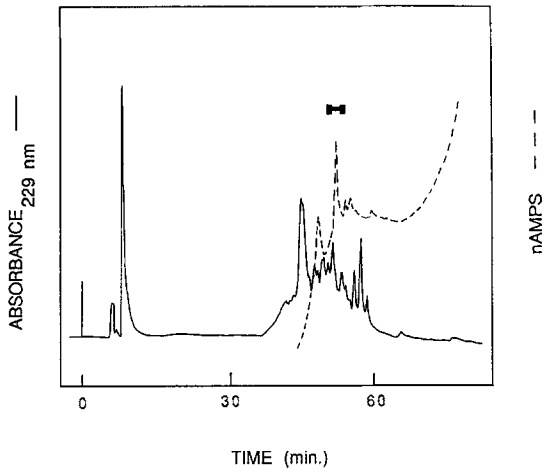

FIGURE 3. Purification of peptide (β212-β215)-(β273-β278) of $(Na^+ + K^+)$ATPase by HPLC on C_4 reverse-phase chromatographic medium. One-tenth each of fractions 42 through 44 from the molecular-exclusion chromatogram were combined, dissolved in 100 μL 1% acetic acid, 0.2% trifluoroacetic acid, and injected onto the Vydac C_4 column equilibrated in 0.1M sodium chloroacetate, pH 5.0. The sample was eluted with a linear gradient between 0.1M sodium chloroacetate, pH 5.0 and 60% n-propanol, 40% 0.1M sodium chloroacetate, pH 5.0 developed over 120 min at a flow rate of 0.5 mL min^{-1}. Absorbance at 280 nm was monitored at 0.1 absorbance units full scale and electrochemical detection was performed at a setting of 50 nanoamperes full scale.

Cystine-containing Peptide (β212-β215)-(β273-β278) of $(Na^+ + K^+)$ATPase. The pool of the fractions from the column of Bio-gel A5m containing the α polypeptide of acetylcholine receptor (distribution coefficient of 0.60) were digested with 10 μg thermolysin $(mg\ protein)^{-1}$. A second, equal addition of thermolysin was made after six hours, and the digestion was allowed to continue overnight. This digestion was terminated by lyophilization, and the resulting peptides (14 mg of protein as determined by amino acid analysis) were dissolved in 88% formic acid:95% ethanol::3:1 (v/v) and submitted to molecular-exclusion chromatography on the column of Fractogel TSK HW40s equilibrated in this solvent. The chromatogram was developed at a flow rate of 4.8 mL h^{-1}, and fractions of 0.8 mL were collected. Fractions from this chromatogram containing cystine (Fractions 41 through 44; distribution coefficients 0.64 to 0.72) were submitted one at a time to HPLC on the C_4 reverse-phase column developed with a linear gradient between 0.1M sodium chloroacetate, pH 5.0 and 40% 0.1M sodium chloroacetate, pH 5.0, 60% n-propanol (Figure 3). Several peaks of absorbance at 280nm and electrochemical activity were seen. The fractions designated by the bracket in Figure 3 were combined with the identical fractions from the same chromatographic separation of the remainder of Fractions 42 through 44 from the molecular-exclusion chromatogram. The pool was evaporated, dissolved in 0.3% acetic acid, 0.06% trifluoroacetic acid, and injected onto the Vydac C_4 column equilibrated in 0.1% triflouroacetic acid. The sample was eluted with a linear gradient developed between 0.1% trifluoroacetic acid and 60% isopropanol, 0.04% trifluoroacetic acid developed over 60 min at a flow rate of 0.5 mL min^{-1}. A single peak of electrochemically active peptide eluted at 58 min on this chromatogram. This

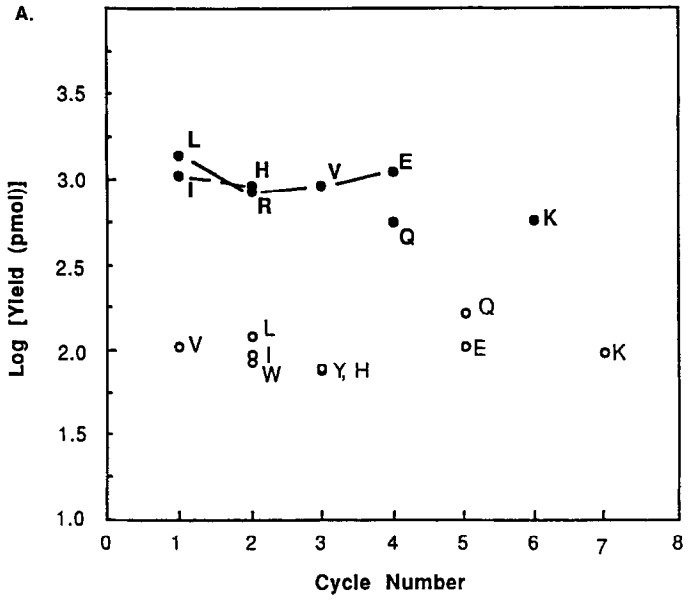

FIGURE 4. Sequence analysis of peptide (β212–β215)–(β273–β278).(A) The logarithms of the yields in picomoles of every phenylthiohydantoin of an amino acid observed on a particular cycle of the automated sequencing are plotted. The amino acids assigned as those from the designated peptide and those expected at a particular cycle are in bold face. (B) Amino acid sequence of the cystine-containing peptide, (β212–β215)–(β273–β278). The sequence presented is from the complete amino acid sequence of the β polypeptide of (Na^++K^+) ATPase from T. californica (10)and the amino terminus and carboxy terminus were assigned based on the observed sequence and the specificity of the thermolysin used to produce the peptide. Cystine is assumed based on the observed amino acid composition.

peak was collected and submitted to amino acid sequencing (Figure 4A) and amino acid analysis.

The peptide was identified as the thermolytic peptide β212-β215 connected by an intact cystine to the thermolytic peptide β273-β278 of the β polypeptide of (Na^++K^+)ATPase (Figure 4B). With the exception of Glx, which was 30% too low, and Arg, which was 50% too high, the amounts of all of the amino acids expected (Val, Ile, Leu, His, Lys) upon amino acid analysis were within 10% of the stoichiometries in which they appear in this peptide, and those amino acids not expected were present at background levels (≤0.2 nmol for every nmol of peptide). There were 0.4 nmol of cystine observed on the chromatogram of the amino acid analyzer for every nmol of the peptide.

DISCUSSION

The two peptides isolated from purified membranes of electric organ from T. californica each contained a cystine connecting Cysteine 214 and Cysteine 277 of the β polypeptide of (Na^++K^+)ATPase. One, the tryptic peptide (β183-β217)-(β275-β278), contains the potential N-glycosylation site at Asparagine 193, which is homologous to the N-glycosylation site at Asparagine 192 known to be glycosylated in the β polypeptide of canine renal (Na^++K^+)ATPase (11). The lack of the phenylthiohydantoin of asparagine, or the

phenylthiohydantoin of any other amino acid, at cycle eleven of the amino acid sequence of the peptide (β183–β217)–(β275–β278) isolated in this study (Figure 2) is consistent with N-glycosylation at this site. The presence of PTC-glucosamine in the hydrolysate that had been modified with phenylisothiocyanate also indicates glycosylation at some site in this peptide. The presence of a cystine between Cysteine 214 and Cysteine 277 connects covalently two distant locations in the amino acid sequence, and indicates that this segment of the sequence is at an extracellular location. This agrees with models in which only the hydrophobic segment towards the amino terminus spans the membrane, placing the majority of the protein in the extracellular fluid.

AKNOWLEDGMENTS

I thank Dr. Jack Kyte, in whose laboratory this research was done. This research was supported in part by Grant-in-Aid AHA 87-0729 from the American Heart Association, Grant AM-33891 from the National Institutes of Health, and Grant DMB 84-13772 from the National Science Foundation. K.V.K was also supported by Predoctoral Training Grant AM-07233 from the National Institutes of Health.

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