β Subunit of $(Na^+ + K^+)$-ATPase Contains Three Disulfide Bonds†

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ABSTRACT: Previous studies of titratable $(Na^+ + K^+)$-ATPase sulfhydryl groups have indicated the presence of one disulfide bond per mole of holoenzyme. This single disulfide cross-link was assigned to the β subunit on the basis of the difference between the number of titrated "free" sulfhydryl groups and the total number of titrated sulfhydryl groups for each subunit [Esmann, M. (1982) *Biochim. Biophys. Acta 688*, 251; Kawamura, M., & Nagano, K. (1984) *Biochim. Biophys. Acta 694*, 27]. In the present study, β-subunit tryptic peptides containing disulfide cross-links were identified and purified by HPLC. Two new peptides were generated from each disulfide-bonded peptide by reduction with dithiothreitol, and the amino acid compositions of these reduced peptides were determined. The data demonstrate that there are three disulfide bonds in the native β subunit: ^{125}Cys–^{148}Cys, ^{158}Cys–^{174}Cys, and ^{212}Cys–^{275}Cys. The number of disulfide bonds in the β subunit was also estimated by titration of sulfhydryl groups with [^{14}C]iodoacetamide. Six sulfhydryl groups were identified: two sulfhydryl groups were titrated without prior reduction, and four were identified only after reduction of the protein with dithiothreitol. These data, suggesting that the β subunit contains two disulfide bonds, are inconsistent with the peptide isolation experiments, which directly identified three disulfide bonds in the β subunit. This inconsistency was resolved by demonstrating that approximately 20% of each disulfide bond in the β subunit was reduced prior to the start of the experiment, resulting in an underestimation of the number of disulfide-bonded sulfhydryl groups in the β subunit from the titration experiments. Titration of sulfhydryl groups of $(Na^+ + K^+)$-ATPase α subunit with [^{14}C]-iodoacetamide identified 23 total sulfhydryl groups: 19 free sulfhydryl groups and 4 sulfhydryl groups that could be titrated only after reduction of the protein with dithiothreitol. In contrast to previous reports suggesting that there are no disulfide bonds in the α subunit, these data indicate that the α subunit contains two disulfide bonds.

The enzyme $(Na^+ + K^+)$-ATPase,[1] also known as the sodium pump, consists of two noncovalently linked subunits, α and β (Jorgensen, 1982). All known functional sites of the holoenzyme, including the nucleotide binding site (Farley et al., 1984) and the cardiac glycoside binding site (Takeyasu, 1988), are located on the α-subunit polypeptide (MW = 112000). The β-subunit polypeptide of dog kidney $(Na^+ + K^+)$-ATPase is a membrane-embedded glycoprotein (protein MW = 35000) (Brown et al., 1987) with three N-linked oligosaccharide modifications (Miller & Farley, 1988). The function of the β-subunit polypeptide is unknown, although it is thought that the β subunit may play an important role in the biosynthesis or assembly of functional pump molecules (Geering et al., 1985; Noguchi et al., 1987).

Sulfhydryl groups of $(Na^+ + K^+)$-ATPase have been extensively studied. Modification of free sulfhydryl groups in the α subunit of $(Na^+ + K^+)$-ATPase has been found to inactivate the enzyme (Winslow, 1981), and it has been suggested that reduction of a disulfide bond in the β subunit also inactivates the $(Na^+ + K^+)$-ATPase (Kawamura & Nagano, 1984). Previous studies of titratable $(Na^+ + K^+)$-ATPase sulfhydryl groups have indicated that there is only one disulfide bond per mole of holoenzyme. This single disulfide cross-link was assigned to the β subunit on the basis of the difference between the number of titrated "free" sulfhydryl groups and the total number of titrated sulfhydryl groups for each subunit (Esmann, 1982; Kawamura & Nagano, 1985). The total sulfhydryl group content of the α and the β subunits determined in these experiments, however, is approximately 40% lower than the number known for each subunit form their amino acid sequences (Brown et al., 1987; Shull et al., 1986a). This observation suggests that previous estimates of α-subunit and β-subunit sulfhydryl groups and disulfide bonds were inaccurate due to the incomplete titration of all sulfhydryl groups.

The dog kidney β subunit contains seven cysteine residues (^{44}Cys, ^{125}Cys, ^{148}Cys, ^{158}Cys, ^{174}Cys, ^{212}Cys, and ^{275}Cys), and the possibility exists for three intramolecular disulfide bonds. There is no evidence for covalent intermolecular associations between $(Na^+ + K^+)$-ATPase subunits. Recent peptide isolation experiments have demonstrated that a disulfide cross-link exists between ^{158}Cys and ^{174}Cys in the dog kidney β subunit (Ohta et al., 1986; Miller & Farley, 1988). A second disulfide bond involving ^{275}Cys and an unidentified cysteine was inferred from the mobility of chymotryptic fragments of the β subunit on polyacrylamide gels in the presence or absence of reducing agents (Farley et al., 1986; Brown et al., 1987). In the present study, tryptic peptides containing disulfide cross-links were identified and purified from $(Na^+ + K^+)$-

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[1] Abbreviations: $(Na^+ + K^+)$-ATPase, sodium- and potassium-dependent adenosinetriphosphatase; DTT, dithiothreitol; TPCK, tosylphenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; aufs, absorbance units full scale.

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ATPase by HPLC. Reduction of each peptide with dithiothreitol generated two new peptides from each original peptide, and the amino acid compositions of these reduced peptides indicate the presence of three disulfide bonds in the β subunit: ^{125}Cys–^{148}Cys, ^{158}Cys–^{174}Cys, and ^{212}Cys–^{275}Cys. In addition to the determination of the number of disulfide bonds in the $(Na^+ + K^+)$-ATPase β subunit by isolation and reduction of disulfide-bonded peptides, the number of free and disulfide-bonded sulfhydryl groups of both the α subunit and the β subunit were estimated by titration with [^{14}C]iodoacetamide. A total of 23 sulfhydryl groups were titrated in the dog kidney α subunit, in quantitative agreement with the total number of Cys residues deduced from the cDNA sequence of sheep kidney (Shull et al., 1986a) and pig kidney (Ovchinnikov, 1986) $(Na^+ + K^+)$-ATPase α subunits. In contrast to previous reports, however, the data reported here indicate that the $(Na^+ + K^+)$-ATPase α subunit contains two disulfide bonds.

Materials and Methods

Materials. Iodoacetamide was purchased from Aldrich. [^{14}C]Iodoacetamide (24.1 mCi/mmol) was purchased from Du Pont-NEN and was diluted with nonradioactive iodoacetamide to 117 μCi/mmol for the titration experiments and to 399 μCi/mmol for the peptide isolation experiments. DTT and TPCK-treated trypsin were obtained from Sigma Chemical, SDS was obtained from Bio-Rad, and ultrapure urea was obtained from Bethesda Research Laboratories. HPLC-grade solvents were obtained from VWR. Trifluoroacetic acid, amino acid standards, triethylamine, phenyl isothiocyanate, and vaccuum hydrolysis tubes were obtained from Pierce Chemical Co. Glycopeptidase A was obtained from ICN.

Purification of $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was purified from dog kidney outer medulla by the method of Jorgensen (1974). Ouabain-sensitive ATPase activity of the purified enzyme was 22.7 ± 0.5 μmol of ATP hydrolyzed mg^{-1} min^{-1}. Protein determinations were made according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Bovine serum albumin concentrations were determined spectrophotometrically with a molar extinction coefficient of 1.38 cm^{-1} at 279 nm for a 0.20% (w/v) solution of protein.

Titration of $(Na^+ + K^+)$-ATPase Sulfhydryl Groups by ^{14}C-Alkylation and Purification of the Labeled $(Na^+ + K^+)$-ATPase α and β Subunits. In order to quantitate the number of free and disulfide-bonded sulfhydryl groups of $(Na^+ + K^+)$-ATPase, the enzyme was radioactively S-alkylated with [^{14}C]iodoacetamide either before or after reduction with DTT. $(Na^+ + K^+)$-ATPase was alkylated with [^{14}C]iodoacetamide, followed by reduction and then a second alkylation with nonradioactive iodoacetamide to exclusively label free sulfhydryl groups. To specifically label disulfide-bonded sulfhydryl groups, $(Na^+ + K^+)$-ATPase was alkylated with nonradioactive iodoacetamide in order to protect the free sulfhydryl groups and then reduced and alkylated with [^{14}C]iodoacetamide. A total of 3.0 mg of purified $(Na^+ + K^+)$-ATPase was denatured in 1.5 mL of 0.2% (w/v) Na_2EDTA, 2.0% (w/v) SDS, 8 M urea, and 200 mM Tris-HCl, pH 8.2, at 37 °C for 15 min. Either radiolabeled or nonradiolabeled iodoacetamide in approximately 150 μL of 100% ethanol was added to a final concentration of 30 mM, and the sample was immediately degassed under vacuum and flushed with nitrogen. Alkylation was allowed to proceed in the dark under a nitrogen atmosphere at room temperature for 60 min. The alkylated protein was separated from iodoacetamide on a 20 cm × 0.8 cm column of Sephadex G-50 (fine) resin, eluted with 0.2% (w/v) Na_2EDTA, 0.2% (w/v) SDS, and 200 mM Tris-HCl, pH 8.2. The protein fraction was collected and concentrated to a volume of 1.0 mL under a stream of nitrogen gas, and solid urea was added to 8 M. The alkylated protein was then reduced by adding solid DTT to 50 mM and incubating at room temperature in a nitrogen atmosphere for 60 min. The reduced protein was separated from excess DTT by desalting on a Sephadex G-50 column as described above, with 0.5 mM DTT included in the elution buffer. The protein fraction was collected and concentrated to a volume of 1.0 mL under a stream of nitrogen gas, and solid urea was added to 8 M. The sample was subjected to a second alkylation, with either radiolabeled or nonradiolabeled iodoacetamide, as described above. The completely reduced and alkylated protein was then desalted a final time as described above.

To label all of the sulfhydryl groups of $(Na^+ + K^+)$-ATPase in a third sample, the alkylation prior to reduction was omitted, and the protein was completely reduced, desalted, and then radioactively S-alkylated as described above.

The α and β subunits of $(Na^+ + K^+)$-ATPase were separated over a 120 cm × 1.5 cm column of Sepharose 4B eluted with 1 mM Na_2EDTA, 0.2% (w/v) SDS, and 20 mM Tris-HCl, pH 8.0. α-Subunit and β-subunit pools were lyophilized and dialyzed for 48 h at 4 °C against 2 L of 5 mM Tris-HCl, pH 7.0, with one change of buffer. Sample volumes were reduced to 1.0 mL under a stream of nitrogen gas and then analyzed for protein content by the method of Lowry et al. (1951). Radioactivity of samples (dpm/mg of protein) was measured by scintillation counting. Aliquots of purified α- and β-subunit samples were homogeneous as determined on 10% Laemmli (1970) polyacrylamide slab gels.

Labeling of $(Na^+ + K^+)$-ATPase Sulfhydryl Groups by ^{14}C-Alkylation, Purification of β Subunit, and Identification of Sulfhydryl Peptides and Disulfide Peptides. In order to identify peptides containing free sulfhydryl groups and peptides containing disulfide cross-links in trypsin digests of purified β subunit, the following labeling strategy was employed. To label only sulfhydryl groups involved in disulfide bonds, a 5.0-mg sample of $(Na^+ + K^+)$-ATPase was S-alkylated with nonradiolabeled iodoacetamide and then reduced and alkylated with [^{14}C]iodoacetamide as described above. This reduced and radioactively labeled sample was designated "R". A second 5.0-mg sample of $(Na^+ + K^+)$-ATPase was subjected to [^{14}C]iodoacetamide alkylation only, without subsequent reduction, in order label free sulfhydryl groups and to preserve the native disulfide cross-links. This nonreduced sample was designated "NR". The β subunits of each sample were purified as described above. After dialysis, the sample volume was reduced to 1.0 mL under a stream of nitrogen gas, and 0.5 mL of dry Dowex AG1-X8 regenerated from 50 mM sodium phosphate (pH 7.0) was added to remove any remaining SDS. The samples were then spun in a clinical centrifuge, and the SDS-free protein solution was removed with a Pasteur pipet.

Purified, SDS-free R and NR β-subunit samples were exhaustively digested with TPCK-treated trypsin in 50 mM Tris-HCl, pH 7.0, at a β-subunit:trypsin weight ratio of 10:1 for 16 h at 37 °C.

Approximately 100 μg of R β-subunit tryptic peptides and 200 μg of NR β-subunit tryptic peptides were separated on a Vydac C_{18} TP218 HPLC column as described in Figure 1. Fractions of 30 s were collected and counted for radioactivity. Peptides containing sulfhydryl groups originally involved in β-subunit disulfide bonds were readily identified in the chromatogram of the R β-subunit tryptic digest by coincident peaks of UV absorbance and radioactivity. Tryptic disulfide peptides from the NR β-subunit digest that were likely to contain

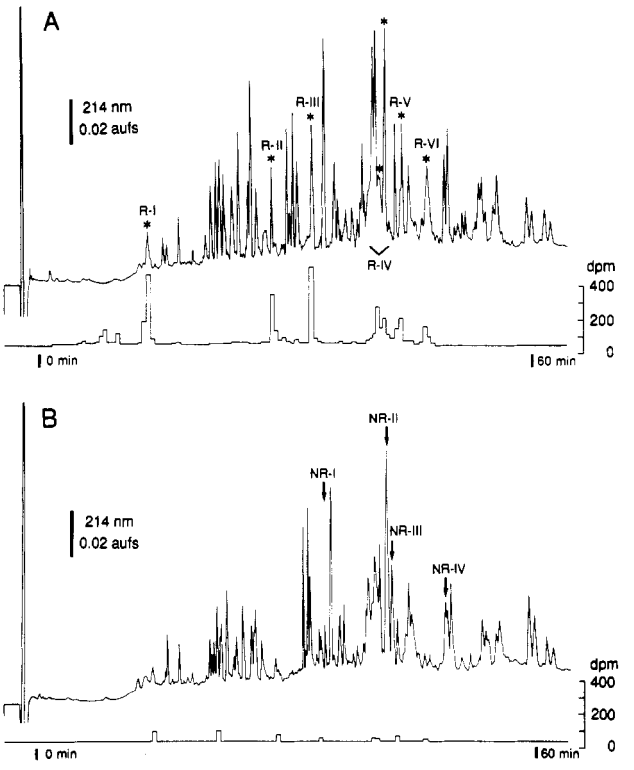

FIGURE 1: HPLC separations of β-subunit tryptic peptides. R and NR β-subunit tryptic digests were separated on a Vydac C_{18} HPLC column (buffer A, 0.1% TFA; buffer B, 0.1% TFA in 95/5 acetonitrile/H_2O; gradient, 0%–60% in 90 min started 4 min after injection; flow rate, 1.5 mL/min). (A) R β-subunit tryptic peptide HPLC chromatogram. Six radioactive fractions from the R β-subunit tryptic peptide HPLC separation were collected (R-1 to R-VI, radioactive peaks indicated by astrisks). These fractions were repurified on HPLC using a shallow gradient (0%–60% buffer B in 270 min; second purification chromatograms not shown). Seven radioactive peaks were purified (R-I-3, R-I-4, R-II-1, R-III-1, R-IV-1, R-IV-2, and R-V-1). The yield from fraction R-VI was very low, and this fraction was not analyzed further. Peptide R-IV-2-G1, the nonglycosylated form of R-IV-2, was purified by HPLC after glycopeptidase A treatment of peptide R-IV-2 (chromatogram not shown). (B) NR β-subunit tryptic peptide HPLC chromatogram. Four potential disulfide-cross-linked peptides (designated NR-I to NR-IV, indicated by arrows) were identified as peaks of UV absorbance with no corresponding peak in the R chromatogram (A). These peptides were further purified on HPLC using a shallow solvent gradient (0%–90% buffer B in 270 min; second purification chromatograms not shown). Glycopeptidase A treatment of peptide NR-IV and subsequent HPLC separation resulted in the purification of peptide NR-IV-G1, the nonglycosylated form of NR-IV (chromatogram not shown).

disulfide cross-links were identified as peaks of UV absorbance in the NR chromatogram that had no corresponding peak in the R chromatogram.

Purification of Radioactive Peptides. A 1.5-mg R β-subunit tryptic digest was separated by HPLC (Figure 1), and six radioactive fractions, designed R-I to R-VI, were collected. Radioactive fractions were subjected to a second HPLC separation using a shallow gradient, and individual peaks were collected. Radioactive peaks were identified by scintillation counting of fraction aliquots. By this method, radioactive peptides R-I-3, R-I-4, R-II-1, R-III-1, R-IV-1, R-IV-2, and R-V-1 were isolated. HPLC separation of radioactive fraction R-VI resulted in very poor recovery of peptides and radioactivity, and this fraction was not analyzed further. Radioactive peptide R-IV-2 exhibited a broad peak shape characteristic of a glycopeptide. To determine whether this peptide was a glycopeptide, the sample was reacted for 16 h at 37 °C with 50 units of glycopeptidase A in 50 μL of 50 mM sodium citrate adjusted to pH 5.5 with phosphoric acid. The sample was subjected to HPLC separation, and a new radioactive peak with a narrow line shape, designated R-IV-2-G1, was collected after the original peak. This behavior is characteristic of glycopeptides (Miller & Farley, 1988).

Purification of Disulfide-Containing Peptides. HPLC chromatograms of the NR and R β-subunit tryptic peptides were compared by overlaying one chromatogram with the other on a lightbox. Four peaks of UV absorbance in the NR chromatogram which had no corresponding peak in the R chromatogram were identified (Figure 1). These peaks were collected from an HPLC separation of 1.5 mg of NR β-subunit tryptic digest and were further purified by a second HPLC separation using a shallow gradient. These purified peptides were designated NR-I-1, NR-II-1, NR-III-1, and NR-IV-1. As with peptide R-IV-2, peptide NR-IV-1 exhibited a broad peak shape typical of a glycopeptide, and this peptide was reacted with glycopeptidase A as described above. A new peptide, designated NR-IV-1-G1, was purified by HPLC after glycopeptidase A treatment of NR-IV-1. To determine if NR-I-1, NR-II-1, NR-III-1, and NR-IV-1-G1 contained disulfide cross-links, each peptide was incubated in 30 μL of 25 mM DTT, 0.2% (w/v) Na_2EDTA, and 200 mM Tris-HCl, pH 8.2, for 60 min at 37 °C. Each reduced sample was then subjected to HPLC separation, and in all cases DTT treatment resulted in the disappearance or reduction in abundance of the original peak and the appearance of two new peaks in the chromatogram (Figure 2). The eight new peptides were collected and designated NR-I-I A and B, NR-II-1 A and B, NR-III-1 A and B, and NR-IV-1-G1 A and B. These peptides were hydrolyzed for amino acid analysis as described below without prior S-alkylation.

Amino Acid Composition Analysis of Purified Peptides. All purified peptides were hydrolyzed in 6 N HCl for 24 h at 110 °C in Pierce vacuum hydrolysis tubes. Samples were degassed under vacuum and sealed prior to hydrolysis. Hydrolysates were dried in a SpeedVac (Savant Instruments) for 2 h and then were derivatized with phenyl isothiocyanate according to the method of Ebert (1986). Phenylthiocarbamyl derivatives of amino acids were separated as described by Ebert (1986) on a Spherisorb ODS-2 C_{18} HPLC column (Thomson Instrument Co.) and detected at 254 nm with a Waters Associates 441 detector. Peak areas were measured with a Shimadzu peak integrator. Recoveries of phenylthiocarbamyl amino acids from hydrolysates were quantitated with the computer program AAQUANT (Miller & Farley, 1989).

After hydrolysis it was determined that air had leaked into some of the hydrolysis tubes, and the quantitation of Cys was unreliable. Consequently, this amino acid was not used in the analysis to determine the identities of the peptides.

Identification of β-Subunit Proteolytic Peptides. Because of the possible contamination of trypsin with chymotryptic activity, all possible peptides within the dog kidney β-subunit amino acid sequence (Brown et al., 1987) that could be generated by cleavage of the polypeptide with either trypsin or chymotrypsin were identified by the DIGEST routine in AAQUANT (Miller & Farley, 1989). Peptides generated by digestion with either trypsin, chymotrypsin, or both proteases were included in the analysis. The amino acid composition of each proteolytic peptide identified by this search was compared to the amino acid compositions of the experimental samples with the FINDCOMP routine in AAQUANT (Miller & Farley, 1989). Proteolytic peptides of the β subunit with more than 50% amino acid composition homology with any experimental peptide are listed in Tables I and III. The percent amino acid homology between each experimental peptide and

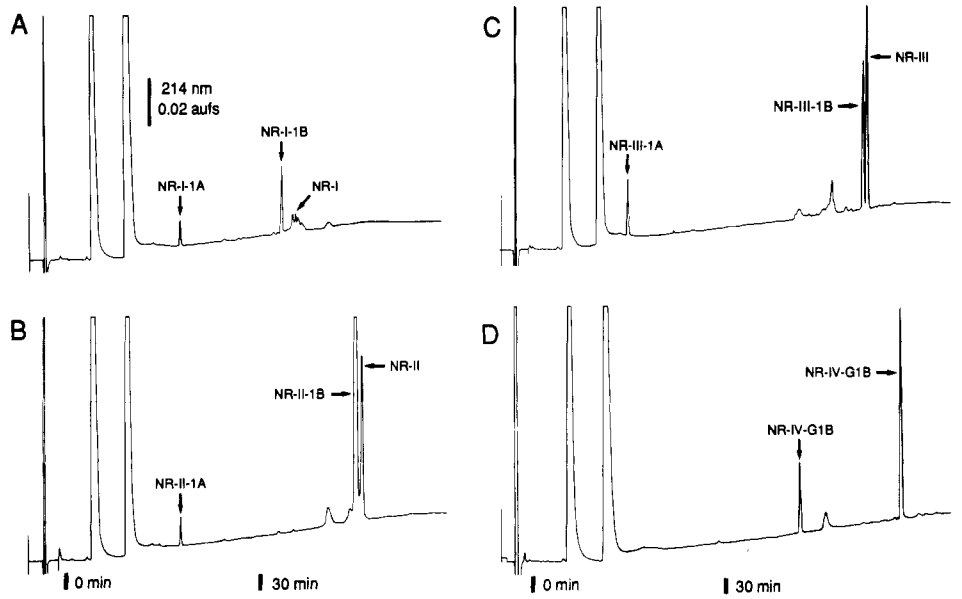

FIGURE 2: Reduction of disulfide-cross-linked NR β-subunit peptides. Purified β-subunit peptides NR-I, NR-II, NR-III, and NR-IV-G1 (Figure 1B) were incubated in 30 μL with 25 mM DTT and 100 mM Tris-HCl, pH 8.0, for 60 min at 37 °C and immediately separated on HPLC. Two new peptides, designated A/B pairs, were produced by reduction of each original peptide. The new peaks were collected for amino acid composition analysis. Panels A–D, chromatograms of reduced peptides NR-I to NR-IV-G1, respectively. HPLC conditions: same as described in Figure 1. The two large peaks eluting early in each chromatogram are unique to DTT.

the proteolytic peptides is also indicated in Tables I and III. This percentage was obtained from

$$[1 - (D/T)] \times 100$$

where D is the number of amino acids that are inconsistent between the compositions of the proteolytic peptide and the experimental peptide and T is the number of amino acids in the proteolytic peptide. Cys was not included in the calculation. Because the experimental peptides had either been labeled with [^{14}C]iodoacetamide or had been generated as a result of dithiothreitol reduction of a precursor peptide, however, the calculation was also performed including Cys. The percentage homology obtained after inclusion of Cys in the calculation is indicated in parentheses in Tables I and III.

Results

Purification and Identification of β-Subunit Tryptic Peptides Containing Sulfhydryl Groups Involved in Disulfide Cross-Links and Assignment of β-Subunit Disulfide Bonds. Radioactive S-alkylation of β-subunit sulfhydryl groups involved in disulfide cross-links was accomplished by first S-alkylating free sulfhydryl groups of the denatured holoenzyme with nonradiolabeled iodoacetamide, followed by complete reduction, and then S-alkylation of the protein with [^{14}C]-iodoacetamide. The β subunit labeled in this procedure (designated R β subunit) was exhaustively digested with trypsin, and seven radioactive peptides were identified after HPLC separation (Figure 1A). Six chromatographic fractions containing the radioactive peptides were pooled as indicated in Figure 1A, and seven radioactive peptides were purified by rechromatography of each pool. One of the peptides, R-IV-2, showed a broad peak shape upon HPLC purification, suggesting it may contain an oligosaccharide modification. Glycopeptidase A treatment of this peptide and subsequent HPLC separation resulted in the purification of a new radioactive peptide, R-IV-2-G1, and a decrease in the abundance of the original peptide (Miller & Farley, 1988).

Amino acid composition analysis of each radioactive peptide was used to identify the corresponding Cys residue within in the amino acid sequence of the β subunit (Brown et al., 1987). All of the tryptic and/or chymotryptic peptides of the β subunit whose amino acid composition are more than 50% homologous to the composition of the radioactive peptides are listed in Table I, together with the percentage homology to each radioactive peptide. The identity of each radioactive peptide within the β-subunit sequence was determined by the extent of homology to the proteolytic peptides. Although Cys was not included in this analysis, the peptides identified by this procedure include sequences containing six of the seven β-subunit sulfhydryl groups. The seventh Cys is located in a very hydrophobic tryptic fragment that was not recovered in this experiment. Two tryptic peptides within the β-subunit sequence were identified with equal probability for peptide R-I-4. These sequences (I-E-C-K and I-E-V-K) represent amino acids 273–276 and 298–301, respectively. The sequence I-E-V-K could be rejected, however, since no Val was present in the hydrolysate. In addition, the amino acid composition of peptide R-I-4 was a perfect match to the sequence 273–276 when Cys was included in the calculation. For certain peptides, such as R-III-1 and R-V-1, the actual site of cleavage by trypsin is not uniquely determined by this analysis because of the sequential occurrence of several protease-sensitive amino acids in the β-subunit sequence. The identification of the Cys residues, however, was not affected by this observation.

Table II lists the amino acid composition of each radioactive peptide, the amino acid composition of the corresponding peptide within the β-subunit sequence, the Cys residue that is found within the peptide, the yield of each peptide, and the radioactive specific activity of each peptide. The variability in peptide recoveries in this experiment is the result of losses due to differences in the efficiency of solubilizing dried HPLC fractions and multiple chromatographic separations and general losses due to handling. The radioactive peptides R-I-1 (^{148}Cys), R-I-2 (^{275}Cys), R-II-1 (^{212}Cys), R-III-1 (^{174}Cys), R-IV-1 (^{212}Cys), and R-IV-2-G1 (^{158}Cys) were recovered with similar specific activities, while peptide R-V-1 (^{125}Cys) was recovered with a specific activity of approximately 25% less than those of the other peptides. The lower specific activity of R-V-1 may, however, be the result of an inaccurate measurement of a small-aliquot volume prior to scintillation

Table I: Identification of Radioactive Cysteine-Containing Peptides of the β Subunit after Amino Acid Analysis[a]

amino acids	R-I-3	amino acids	R-I-4	amino acids	R-II-1	amino acids	R-III-1
2–4	67 (67)	21–23	67 (67)	207–215	100 (100)	169–178	89 (90)
146–149	75 (100)	108–110	67 (67)			170–178	100 (100)
295–297	67 (50)	273–276	75 (100)			173–181	63 (67)
		285–287	67 (67)				
		298–301	75 (50)				

amino acids	R-IV-1	amino acids	R-IV-2-G1	amino acids	R-V-1
207–215	67 (70)	152–166	64 (67)	107–122	54 (47)
				111–133	63 (65)
				112–133	67 (68)
				113–133	70 (71)
				118–133	67 (69)
				118–136	55 (58)
				122–138	56 (59)

[a] Radioactive R peptides were hydrolyzed in 6 N HCl for 24 h in Pierce vacuum hydrolysis tubes. Amino acid compositions were determined from PTC-amino acids (Ebert, 1986) and were quantitated with the computer program AAQUANT (Miller & Farley, 1989). The amino acid compositions were compared to the theoretical compositions of all peptides within the dog kidney β subunit that could be generated by cleavage of the β subunit by trypsin and/or chymotrypsin. The percent homology between experimental and theoretical peptides was calculated from the expression $[1 - (D/T)] \times 100$, where D/T is the fraction of amino acids in the theoretical peptide that are different from the experimental sample. The theoretical peptides with greater than 50% homology to each R peptide are indicated by the first and last amino acids of the peptide within the β-subunit sequence. The percent homology between each theoretical peptide and the R peptides is indicated under each R peptide. Cys and Trp were not included in the analysis. The numbers in parentheses are the percent homology obtained when Cys is included in the calculation.

Table II: Amino Acid Composition[a] of Radioactive Cysteine-Containing Peptides in the β-Subunit Amino Acid Sequence[b]

	peptide						
amino acid	R-I-3	R-I-4	R-II-1	R-III-1	R-IV-1	R-IV-2-G1	R-V-1
Ala	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.8 (1)
Arg	1.1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (1)	0 (0)
Asx	0 (0)	0 (0)	0 (0)	0.9 (1)	0 (0)	1.7 (3)	2. 0 (4)
Cys	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)
Glx	0 (0)	1.0 (1)	1.1 (1)	0 (0)	0.9 (1)	2.2 (2)	2.2 (4)
Gly	0 (0)	0 (0)	1.0 (1)	1.1 (1)	1.0 (1)	3.6 (2)	1.0 (1)
His	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ile	0 (0)	1.0 (1)	0 (0)	1.1 (1)	0 (0)	1.0 (1)	1.6 (2)
Leu	0 (0)	0 (0)	1.1 (1)	1.2 (1)	1.2 (1)	1.9 (2)	0 (0)
Lys	1.1 (1)	1.0 (1)	0.8 (1)	1.8 (2)	0.8 (1)	0 (0)	1.5 (2)
Met	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.7 (2)
Phe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.7 (1)
Pro	0 (0)	0 (0)	0.8 (1)	1.0 (1)	1.4 (1)	0 (0)	0.8 (1)
Ser	0 (0)	0 (0)	0 (0)	0 (0)	0.7 (0)	1.3 (1)	1.3 (2)
Thr	0 (0)	0 (0)	0.7 (1)	0 (0)	0.8 (1)	1.7 (2)	0 (0)
Tyr	0 (0)	0 (0)	0 (0)	0 (0)	0.6 (0)	0 (1)	0 (0)
Val	1.0 (1)	0 (0)	1.5 (1)	1.0 (1)	1.4 (1)	0 (0)	0 (0)
Cys[c]	^{148}Cys	^{275}Cys	^{212}Cys	^{174}Cys	^{212}Cys	^{158}Cys	^{125}Cys
recovery[d]	1.27	3.12	1.80	3.88	1.65	0.40	1.20
sp act.[e]	0.78	0.77	0.81	0.85	0.86	0.79	0.59

[a] PTC-amino acids were quantitated with the computer program AAQUANT (Miller & Farley, 1989) and are expressed as moles per mole of peptide. Hydrolysis-resistant amino acids Val, Ile, and Gly were used to normalize each sample. The theoretical amino acid composition of the corresponding peptide derived from the amino acid sequence (Brown et al., 1987) is shown in parentheses. The identity of the R peptides was determined from the data in Table I as follows [peptide (amino acids)]: R-I-3 (146–149); R-I-4 (273–276); R-II-1 (207–215); R-III-1 (170–178); R-IV-1 (207–215); R-IV-2-G1 (152–166); R-V-1 (113–133). Cys was not determined in this analysis (nd) because of oxidative destruction. [b] Radioactive R peptides were hydrolyzed in 6 N HCl for 24 h in Pierce vacuum hydrolysis tubes. Hydrolysates were derivatized with phenyl isothiocyanate, and PTC-amino acids were separated on HPLC (Ebert, 1986). [c] Peptides containing six of the seven β-subunit sulfhydryl groups were recovered in this experiment. The Cys residue contained within each peptide is listed. Peptide R-IV-1 was cleaved after ^{206}Tyr to yield peptide R-II-1, and peptide R-IV-2-G1 was cleaved by trypsin between ^{168}Tyr and ^{169}Arg, suggesting that there was some chymotryptic activity in the trypsin. [d] The radioactivity (dpm) of each peptide sample was measured by scintillation spectroscopy, and the yield (nmol) of each is listed. 10%–25% of each peptide was used for amino acid composition analysis. [e] The specific activity of each radioactive peptide is expressed as moles of ^{14}C per mole of peptide.

counting. Because the specific activities of the sulfhydryl groups labeled by this procedure should occur in pairs (Mise & Bahl, 1980), it is not likely that the low specific activity observed with peptide R-V-1 reflects a unique property of ^{125}Cys. The amino acid immediately preceding ^{207}Val at the amino terminus of peptide R-II-1 is Tyr and suggests that the trypsin used in these experiments also contained a small amount of chymotryptic activity. This accounts for the appearance of ^{212}Cys in peptides R-II-1 and R-IV-1, which differ only by the presence of the amino acids Y-S-P-Y at the amino terminus of peptide R-IV-1.

Identification of six radioactive Cys residues with similar specific activities from the R β subunit suggested that the dog kidney $(Na^+ + K^+)$-ATPase β subunit contains three disulfide bonds. The specific activity for each of the seven R peptides was about 0.8 mol of ^{14}C/mol of peptide, indicating that approximately 80% of each peptide was S-alkylated by [^{14}C]iodoacetamide and 20% had been S-alkylated by nonradiolabeled iodoacetamide before reduction. As discussed below, this labeling pattern may be explained if approximately 20% of each β-subunit disulfide bond was reduced prior to nonradioactive S-alkylation of the holoenzyme. This reduction may have occurred during the purification of $(Na^+ + K^+)$-ATPase and subsequent storage or handling.

In order to determine the arrangement of the different disulfide cross-links of the β subunit and to test the suggestion

Table III: Identification of Disulfide-Bonded Peptides within the β Subunit after Amino Acid Analysis[a]

amino acids	NR-I-1A	amino acids	NR-I-1B	amino acids	NR-II-1A	amino acids	NR-II-1B
21–23	67 (67)	207–215	100 (100)	21–23	67 (67)	203–215	91 (92)
108–110	67 (67)			108–110	67 (67)		
273–276	75 (100)			273–276	75 (100)		
285–287	67 (67)			285–287	67 (67)		
298–301	75 (50)			298–301	75 (50)		
amino acids	NR-III-1A	amino acids	NR-III-1B	amino acids	NR-IV-G1A	amino acids	NR-IV-G1B
2–4	67 (67)	111–133	86 (87)	170–178	75 (78)	152–168	69 (71)
146–149	75 (100)	112–133	90 (91)	182–188	63 (56)		
295–297	67 (50)	113–133	85 (86)				
		118–133	60 (63)				
		118–135	65 (67)				
		123–139	56 (59)				

[a] Amino acid compositions of A/B peptide pairs generated by dithiothreitol reduction of putative disulfide-bonded peptides were obtained from PTC-amino acids (Ebert, 1986) after acid hydrolysis. The amino acid compositions were compared to the amino acid compositions of theoretical peptides within the β subunit as described in the legend to Table I. The percent homology of each theoretical peptide with the A or B peptide is indicated in the table. The number in parentheses is the percent homology calculated when Cys is included in the analysis.

that a fraction of each β-subunit disulfide bond was reduced prior to the experiment, a second sample of $(Na^+ + K^+)$-ATPase (designated NR) was S-alkylated with [^{14}C]iodoacetamide in order to label free sulfhydryl groups, but this sample was not reduced in order that the disulfide bonds would remain intact. The purified NR β subunit was digested with trypsin, and the peptides were separated by HPLC (Figure 1B). The chromatogram of NR β-subunit tryptic peptides displays a radioactivity profile nearly identical with the R β-subunit tryptic peptide chromatogram, except that radioactive peaks in the NR chromatogram are only about 20%–25% as large as the corresponding R chromatogram peaks. This observation confirms the previous inference that prior to reduction of the protein with DTT approximately 20% of all disulfide-bonded sulfhydryl groups of the β subunit were available for alkylation. Four new UV absorbance peaks with no corresponding peak in the R chromatogram were observed in the NR chromatogram. These new peaks, designed NR-I, NR-II, NR-III, and NR-IV, were purified further. Peptide NR-IV appeared to have a peak shape characteristic of a glycopeptide (Miller & Farley, 1988), and glycopeptidase A treatment yielded a new peptide, NR-IV-G1, upon HPLC purification. To determine whether peptides NR-I, NR-II, NR-III, and NR-IV-G1 contained reducible disulfide cross-links, each peptide was incubated with 25 mM DTT and repurified on HPLC. In each case, DTT treatment resulted in the appearance of two new HPLC peaks with retention times different from that of the original peptide and either a decrease in abundance or the complete loss of the original peptide. The eight new peaks, designated as NR-IA/B to NR-IV-G1A/B, were collected and analyzed for amino acid composition. A computer search for these compositions within the dog kidney β-subunit amino acid sequence was performed as before, and the β-subunit peptides whose amino acid compositions are more than 50% homologous with each sample are listed in Table III. The identity of the peptides generated by reduction with DTT was determined on the basis of the percentage amino acid homology with β-subunit peptides, and the amino acid compositions of the experimental samples and the corresponding β-subunit peptides are listed in Table IV. The experimental data are in good agreement with the expected amino acid compositions of tryptic/chymotryptic peptides containing six of the seven Cys residues of the dog kidney β subunit (Brown et al., 1987). Because only one A/B pair of peptides was produced upon reduction of each disulfide-bonded precursor peptide, the identification of these Cys-containing peptides within the β-subunit sequence uniquely determines the locations of three β-subunit disulfide bonds: ^{125}Cys–^{148}Cys, ^{158}Cys–^{174}Cys, and ^{212}Cys–^{275}Cys. The β-subunit tryptic peptides that contain these disulfide bonds are shown at the bottom of Table IV. As indicated previously for peptide R-II-1, the identification of peptides NR-I-1B and NR-II-1B is consistent with the presence of some chymotryptic activity in the trypsin, since the cleavage occurs after ^{206}Tyr and ^{202}Tyr, respectively.

Titration of Free Sulfhydryl Group and Disulfide Sulfhydryl Group Classes of $(Na^+ + K^+)$-ATPase α and β Subunits. Because several previous studies had estimated the total sulfhydryl content of $(Na^+ + K^+)$-ATPase and the number of disulfide bonds from titration studies (Schoot et al., 1978; Esmann, 1982; Kawamura and Nagano, 1984, 1985), it was of interest to measure these parameters in the present study, especially since all of the previous work had been done before the actual number of Cys residues in $(Na^+ + K^+)$-ATPase was known. During the course of this work it was found that the choice of alkylation reagent was critical. In particular, it was possible to titrate 29 of the 30 sulfhydryl groups of $(Na^+ + K^+)$-ATPase with iodoacetamide, but this was not possible with iodoacetic acid. An increased reactivity of haloacetamides relative to their haloacetate counterparts has also been observed by others (Wasserman & Lentz, 1971; Tyson et al., 1989).

Results of the titration experiments are presented in Table V. A total of 23.1 ± 2.5 sulfhydryl groups per mole of α subunit and 5.8 ± 0.25 sulfhydryl groups per mole of β subunit were measured by complete reduction and [^{14}C]iodoacetamide S-alkylation of $(Na^+ + K^+)$-ATPase. The complete amino acid sequence and, therefore, the actual number of Cys residues in dog kidney $(Na^+ + K^+)$-ATPase α subunit are not known; however, both sheep kidney (Shull et al., 1986b) and pig kidney (Ovchinnikov et al., 1986) $(Na^+ + K^+)$-ATPase α subunits are known to contain 23 cysteines. The value of 23.1 ± 2.5 sulfhydryl groups obtained in this experiment for dog kidney $(Na^+ + K^+)$-ATPase α subunit probably reflects the actual number of cysteines in this protein. Dog kidney $(Na^+ + K^+)$-ATPase β subunit contains seven cysteines (Brown et al., 1987); however, in this experiment only 5.8 ± 0.25 sulfhydryl groups per mole of β subunit were measured. This experiment indicated that there are 3.9 ± 0.3 disulfide-bonded sulfhydryl groups in the β subunit, or two disulfide bonds, and 2.4 ± 0.1 free sulfhydryl groups. The discrepancy between the results of the titration experiment and the experiment in which individual disulfide-bonded peptides were identified can be understood from a consideration of the specific activities of the radioactive peptides recovered from the R β-subunit tryptic digest. These data suggest that approximately 20% of each β-subunit disulfide bond per mole of β subunit was reduced

Table IV: Amino Acid Compositions of Disulfide-Bonded Peptides from a Trypsin Digest of (Na^+ + K^+)-ATPase β Subunit[a]

	peptide (β-subunit residues)			
	NR-I-1A (273–276)	NR-I-1B (207–215)	NR-II-1A (273–276)	NR-II-1B (203–215)
Ala	0 (0)	0 (0)	0 (0)	0 (0)
Arg	0 (0)	0 (0)	0 (0)	0 (0)
Asx	0 (0)	0 (0)	0 (0)	0 (0)
Cys	nd (1)	nd (1)	nd (1)	nd (1)
Glx	1.05 ± 0.05 (1)	1.05 ± 0.05 (1)	1.05 ± 0.05 (1)	1.40 ± 0.05 (1)
Gly	0 (0)	1.25 ± 0.05 (1)	0 (0)	1.40 ± 0.10 (1)
His	0 (0)	0 (0)	0 (0)	0 (0)
Ile	1.0 (*) (1)	0 (0)	1.0 (*) (1)	0 (0)
Leu	0 (0)	1.0 (*) (1)	0 (0)	1.15 ± 0.10 (1)
Lys	1.08 ± 0.02 (1)	0.98 ± 0.02 (1)	0.95 ± 0.05 (1)	1.15 ± 0.05 (1)
Met	0 (0)	0 (0)	0 (0)	0 (0)
Phe	0 (0)	0 (0)	0 (0)	0 (0)
Pro	0 (0)	1.05 ± 0.05 (1)	0 (0)	2.00 ± 0.02 (2)
Ser	0 (0)	0 (0)	0 (0)	1.05 ± 0.05 (1)
Thr	0 (0)	1.05 ± 0.05 (1)	0 (0)	1.05 ± 0.05 (1)
Trp	0 (0)	0 (0)	0 (0)	0 (0)
Tyr	0 (0)	0 (0)	0 (0)	0.85 ± 0.05 (2)
Val	0 (0)	2.15 ± 0.05 (2)	0 (0)	2.0 (*) (2)

NR-I-1A IECK ^{275}Cys
NR-I-1B VLPVQCTGK ^{212}Cys

NR-II-1A IECK ^{275}Cys
NR-II-1B YSPYVLPVQCTGK ^{212}Cys

	peptide (β-subunit residues)			
	NR-III-1A (146–149)	NR-III-1B (112–133)	NR-IV-G1A (170–178)	NR-IV-G1B (152–168)
Ala	0 (0)	1.05 ± 0.15 (1)	0 (0)	0 (0)
Arg	1.22 ± 0.02 (1)	0 (0)	0 (0)	0 (0)
Asx	0 (0)	3.30 ± 0.40 (4)	0 (1)	2.65 ± 0.35 (3)
Cys	nd (1)	nd (1)	nd (1)	nd (1)
Glx	0 (0)	3.60 ± 0.40 (4)	0 (0)	2.75 ± 0.25 (2)
Gly	0 (0)	1.20 ± 0.20 (1)	2.10 ± 0.50 (1)	4.60 ± 0.7 (3)
His	0 (0)	0 (0)	0 (0)	0 (0)
Ile	0 (0)	1.70 ± 0.10 (2)	1.0 (*) (1)	1.0 (*) (1)
Leu	0 (0)	0 (0)	1.15 ± 0.15 (1)	2.05 ± 0.35 (2)
Lys	1.08 ± 0.02 (1)	2.70 ± 0.30 (3)	1.95 ± 0.35 (2)	0 (0)
Met	0 (0)	1.05 ± 0.05 (2)	0 (0)	0 (0)
Phe	0 (0)	1.0 (*) (1)	0 (0)	0 (0)
Pro	0 (0)	1.00 ± 0.10 (1)	1.25 ± 0.15 (1)	0 (0)
Ser	0 (0)	1.80 ± 0.20 (2)	0 (0)	1.60 ± 0.30 (1)
Thr	0 (0)	0 (0)	0 (0)	1.35 ± 0.15 (1)
Trp	0 (0)	0 (0)	0 (0)	0 (1)
Tyr	0 (0)	0.40 ± 0.10 (0)	0 (0)	1.65 ± 0.15 (2)
Val	1.0 (*) (1)	0 (0)	1.22 ± 0.02 (1)	0 (0)

NR-III-1A KVCR ^{148}Cys
NR-III-1B KDSAQKDEMIFEDCGNMPSEIK ^{125}Cys

NR-IV-G1A DGKPCVLIK ^{174}Cys
NR-IV-G1B LEWLGNCSGINDETYGY ^{158}Cys

[a] Four potential disulfide-cross-linked peptides were purified from a trypsin digest of the nonreduced NR β-subunit sample (designated NR-I, NR-II, NR-III, and NR-IV, Figure 1B). These peptides were repurified by HPLC and reduced in 25 mM DTT. Two new peptides, designated A/B peptide pairs, were produced by reduction of each original peptide and were purified by HPLC (Figure 2). The reduced A/B peptide pairs were analyzed for amino acid composition as described in the legend of Table I. Amino acid recoveries for each peptide were calculated with the computer program AAQUANT (Miller & Farley, 1989) and are presented as moles per mole of peptide (±95% confidence). Peptide recoveries were estimated from the recovery of a hydrolysis-stable amino acid (either Ile, Leu, Phe, or Val, indicated by an asterisk). For each experimentally determined peptide composition, the amino acid composition of the corresponding peptide within the β subunit is shown in parentheses. The identity of each A or B peptide within the β-subunit sequence was determined from the data in Table III and is indicated at the top of the table. Because only one A/B peptide pair was produced upon reduction of NR-I, NR-II, NR-III, and NR-IV-G1, the disulfide cross-links in the original peptides are precisely determined. These disulfide cross-links are indicated in the lower part of the table. The redundancy in peptides NR-I and NR-II is likely to be due to presence of chymotrypsin activity in the trypsin preparation, as discussed in the text.

prior to the labeling experiments. A total of 3.9 ± 0.3 mol of titrated disulfide sulfhydryl groups per mole of β subunit, therefore, probably represent only about 75%–80% of the actual number of disulfide-bonded sulfhydryl groups, which is six. The number of free sulfhydryl groups measured in the titration experiment is higher than expected and also reflects the partial reduction of disulfide bonds prior to labeling. With these considerations, the number of titrated β-subunit disulfide bonds agrees reasonably well with the results of the peptide isolation experiments.

For the α subunit, the number of disulfide-bonded sulfhydryl groups and free sulfhydryl groups per mole of protein were determined in this experiment to be 3.8 ± 0.15 and 18.4 ± 2.4, respectively. Together, these two classes of sulfhydryl groups indicate a total of approximately 23 titratable sulfhydryl groups for the α subunit, in agreement with the DNA sequence. These data suggest that the α subunit contains at least two disulfide cross-links.

Discussion

The results of the peptide isolation experiments described above directly demonstrate that the dog kidney (Na^+ + K^+)-ATPase β subunit contains three disulfide bonds: ^{125}Cys–^{148},Cys, ^{158}Cys–^{174}Cys, and ^{212}Cys–^{275}Cys. The ^{158}Cys–^{174}Cys disulfide cross-link has been identified in two previous reports (Ohta et al., 1986; Miller & Farley, 1988) and is confirmed in the present study. The β subunit is relatively resistant to proteolysis as part of the membrane-bound

Table V: Measurement of $(Na^+ + K^+)$-ATPase Holoenzyme, α-Subunit, and β-Subunit Disulfide Bonds and Free Sulfhydryl Groups by Titration[a]

	mol of sulfhydryl groups/mol of protein								
	disulfide SH			free SH			total SH		
author	α	β	NKA	α	β	NKA	α	β	NKA
this paper[b]	3.8 ± 0.4	3.9 ● 0.3	nd	18.4 ± 3.8	2.4 ± 0.1	nd	23.1 ± 2.5	5.8 ± 0.3	nd
Esmann (1982)[c]	nd	nd	nd	15.0	2.5	nd	13.9	4.2	nd
Kawamura and Nagano (1985)[d]	nd	nd	nd	18.3	2.3	nd	17.0	3.9	nd
Kawamura and Nagano (1984)[e]	nd	nd	nd	nd	nd	17.8	nd	nd	20.0
Schoot et al. (1978)[f]	nd	nd	nd	(15.8)	nd	23.0	nd	nd	nd

[a] Results of five separate studies of $(Na^+ + K^+)$-ATPase sulfhydryl groups. [b] Titration protocol described in the text. Results are presented as the mean ± SD for each determination, $n = 2$. To calculate the molar ratio of titrated sulfhydryl groups per subunit, the following molecular weights were used: α subunit, 112000; β subunit, 35000. [c] Sulfhydryl groups of each subunit were determined by incorporation of 5,5′-dithiobis(2-nitrobenzoic acid), assuming a molecular weight of 106000 for the α subunit and 40000 for the β subunit. [d] Sulfhydryl groups of each subunit were determined by incorporation of *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide, assuming a molecular weight of 104000 for the α subunit and 40000 for the β subunit. [e] Sulfhydryl groups of holoenzyme were determined by incorporation of *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide, assuming a molecular weight of 146000 for the holoenzyme. [f] Free sulfhydryl content of the holoenzyme was determined by incorporation of 5,5′-dithiobis(2-nitrobenzoic acid) [original data recalculated with MW of $(Na^+ + K^+)$-ATPase = 146000]. The value for the α subunit was obtained from the value for the holoenzyme, an α-subunit MW = 112000 being used, on the basis of the observation of Schoot et al. (1978) that essentially all reactive sulfhydryl groups determined in that report were located in the α subunit.

holoenzyme (Farley et al., 1986; Chin, 1985) and as a purified polypeptide (Miller and Farley, unpublished observation), suggesting that this protein retains a compact, well-folded tertiary structure even after denaturation with SDS. The three β-subunit disulfide bonds most likely play an important role in maintaining the stable tertiary structure of this polypeptide. The assignment of dog kidney β-subunit disulfide bonds is probably also valid for the β subunits from sheep kidney (Shull et al., 1986), pig kidney (Ovchinnikov et al., 1986), human HeLa cells (Kawakami et al., 1986), rat kidney and brain (Young et al., 1997), and *Topedo* electroplax (Noguchi et al., 1986), because the number and relative locations of β-subunit sulfhydryl groups from these sources and dog kidney β subunit are 100% conserved.

The results of computer predictions of dog kidney β-subunit secondary structure, described previously (Brown et al., 1987), suggest that the β-subunit secondary structure may be primarily α helical in conformation from the amino terminus until just before the first glycosylation site at ^{157}Asn. The ^{125}Cys–^{148}Cys cross-link occurs in this region. The predicted helical region from amino acids 100 to 125 is an amphipathic structure exhibiting a strong hydrophobic moment as calculated according to Eisenberg (1984) (Miller and Farley, unpublished observation). The amphipathic nature of this region may indicate that the domain is situated at an interface between a hydrophilic surface and a hydrophobic surface, such as the surface of a cell membrane (Kaiser & Kezdy, 1983; Finer-Moore & Stroud, 1984). It is interesting to note that two other regions of the β subunit in the area of residues 130–140 and 145–155 are predicted to be in an α-helical conformation and also appear to possess significant hydrophobic moments. The ^{125}Cys–^{148}Cys disulfide bond may serve to stabilize a domain of three helices that share a common hydrophobic face. The domain between the first and third glycosylation sites, consisting of about 100 amino acids, is predicted as a series of β-sheet and turn regions. The ^{157}Cys–^{174}Cys and ^{212}Cys–^{274}Cys cross-links are located in this domain and may serve to stabilize a compact, highly folded structure in which the three glycosylation sites and a sequence of eight consecutive charged amino acids (amino acids 215–222) are exposed.

As a result of the peptide isolation experiments, six of the seven β-subunit sulfhydryl groups were found to participate in disulfide bonds. No peptide containing ^{44}Cys was identified from either the R β-subunit or the NR β-subunit samples. Assuming that ^{44}Cys does exist as a free sulfhydryl in the β subunit, it was expected that this amino acid would be labeled in the NR sample. ^{44}Cys is located within a tryptic peptide fragment consisting of amino acids 34–64 in the dog kidney β subunit, and this region is thought to represent the membrane-embedded part of the polypeptide (Brown et al., 1987; Kawakami & Nagano, 1988). The large size and extreme hydrophobic nature of this peptide suggest that efficient elution from a C_{18} HPLC column would be unlikely, and this may explain why a peptide containing ^{44}Cys was not recovered in this study. Alternatively, it is possible that ^{44}Cys was not susceptible to S-alkylation by [^{14}C]iodoacetamide. β-Subunit sulfhydryl group titration results indicate that only six of the seven sulfhydryl groups in this polypeptide could be measured by [^{14}C]iodoacetamide alkylation after complete reduction of the protein (Table III), and an inability to alkylate ^{44}Cys with [^{14}C]iodoacetamide is consistent with this possibility.

Although the peptide isolation experiments resulted in the assignment of three β-subunit disulfide bonds, the results of the titration experiments suggested that the β subunit contains only two disulfide bonds. As discussed earlier, this discrepancy may be explained by consideration of the specific activities of the R β-subunit tryptic peptides. These data suggest that approximately 20% of each disulfide bond within the β subunit was reduced prior to the experiment, possibly during purification of the enzyme and handling of the protein. This suggestion was confirmed by the alkylation of the holoenzyme with [^{14}C]iodoacetamide without reduction (Figure 1B). The titrated number of disulfide-bonded sulfhydryl groups actually represents only about 80% of the actual number, which is six (three disulfide bonds). These results illustrate the difficulty of correctly determining the exact number of disulfide bonds by titration experiments that rely solely on quantitating the incorporation of a specific labeling reagent into the protein. For this reason, the α-subunit titration data obtained in the present study, which suggest that there are two disulfide bonds in this polypeptide, will require confirmation by identification of disulfide-bonded α-subunit peptides. Because of the large size of the α subunit and the abundance of cysteine residues in this protein, identifying individual cysteine-containing and disulfide-cross-linked peptides will be difficult.

The titration experiments reported here suggest that the α subunit contains at least two disulfide bonds. This result is in contrast to the previous studies of $(Na^+ + K^+)$-ATPase sulfhydryl groups in which it was concluded that the α subunit lacks disulfide cross-links (Kawamura & Nagano, 1985a,b; Esmann, 1982). One of these studies reported a qualitative

difference in incorporation of a fluorescent alkylating agent into each subunit after labeling reduced and nonreduced holoenzyme (Kawamura & Nagano, 1985a), and two studies attempted to quantitate the number of free sulfhydryl groups and total sulfhydryl groups of each subunit (Kawamura & Nagano, 1985b; Esmann, 1982) (Table V). The estimates of the number of free α-subunit sulfhydryl groups obtained in these studies are similar to the estimate obtained in this paper. The estimates for total sulfhydryl content are much lower in the earlier reports, however, than the known α-subunit sulfhydryl content and also lower than the estimates of free sulfhydryl groups obtained in the same studies (Table V). The data of Esmann (1982) and Kaamura and Nagano (1985b), therefore, may reflect incomplete titrations of α-subunit sulfhydryl groups rather than a lack of disulfide cross-links. During the course of the present study it was found that the nature of the alkylating agent used to titrate the sulfhydryl groups had a profound effect on the number of sulfhydryl groups that could be measured.

It has also been reported that there is one essential disulfide bond in $(Na^+ + K^+)$-ATPase (Kawamura & Nagano, 1984). On the basis of the incorporation of the fluorescent alkylating agent *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide into the α and β subunits following reduction of the enzyme, the essential disulfide bond was assigned to the β subunit. It is possible, however, that reduction of α-subunit disulfides also occurred but that either the resulting sulfhydryl groups were not susceptible to alkylation by that reagent or the increase in α-subunit alkylation was not detectable from the background signal by the fluorescence method employed. Disulfide bonds in the α subunit, the catalytic subunit of $(Na^+ + K^+)$-ATPase, would be likely candidates for any proposed essential disulfide bond, and the titration data presented in this paper suggest that the α subunit may contain two disulfide bonds. Neither the locations of these disulfide bonds within the α subunit nor their importance for $(Na^+ + K^+)$-ATPase activity have been investigated in this study.

Added in Proof

During the review of this paper the results of a separate examination of $(Na^+ + K^+)$-ATPase β-subunit disulfide bonds were published (Kirley, 1989). The locations of disulfide bonds within the β subunit that were determined in that study were identical with the results reported here.

Registry No. ATPase, 9000-83-3.

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