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Tryptic and chymotryptic cleavage sites in sequence of α-subunit of $(Na^+ + K^+)$-ATPase from outer medulla of mammalian kidney

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Localization of selective proteolytic splits in α-subunit of $(Na^+ + K^+)$-ATPase is important for understanding the mechanism of active Na^+,K^+-transport. Proteolytic fragments of α-subunit from pig kidney were purified by chromatography in $NaDodSO_4$ on TSK 3000 SW columns. NH_2-terminal amino acid sequences of fragments as determined in a gas phase sequenator were unambiguously located within the total sequence of α-subunit from sheep kidney (Shull, C.E., et al. (1985) Nature 316, 691–695) and pig kidney (Ovchinnikov, Y.A., et al. (1985) Proc. Acad. Sci. USSR 285, 1490–1495). The primary chymotryptic split in the E_1-form is located between Leu-266 and Ala-267 while the tryptic cleavage site appears to be between Arg-262 and Ile-263 (Bond 3). Tryptic cleavage in the initial fast phase of inactivation of the E_1-form is located between Lys-30 and Glu-31 (Bond 2). In the E_2-form, primary tryptic cleavage is between Arg-438 and Ala-439 (Bond 1). Chymotryptic cleavage between Leu-266 and Ala-267 stabilizes the E_1-form of the protein without affecting the sites for binding of cations or nucleotides. Titration of fluorescence responses demonstrates the importance of the NH_2-terminal for E_1-E_2 transition. Protonation of His-13 facilitates transition from E_1- to E_2-forms of the protein. Removal of His-13 after cleavage of bond 2 can explain the increase in apparent affinity of the cleaved enzyme for Na^+ and the shift in poise of E_1-E_2 equilibrium in direction of E_1-forms. The NH_2-terminal sequence in renal α-subunit is not conserved in α+ from rat neurolemma or in α-subunit from *Torpedo* or brine shrimp. A regulatory function of the NH_2-terminal part of the α-subunit may thus be a unique feature of the α-subunit in $(Na^+ + K^+)$-ATPase from mammalian kidney.

Introduction

In its function as the catalytic protein of the Na^+/K^+ pump, the α-subunit undergoes transitions between E_1 and E_2 conformations that are coupled to cation transfer across the membrane [1]. Residues involved in the conformational transition have been identified by specific proteolytic cleavage. In the E_1-conformation cleavage is restricted to the N-terminal half of the α-subunit, while bonds near the middle and in the C terminal half of the α-subunit are exposed to cleavage in the E_2-form [1–3]. The consequences of these splits for cation transport and catalytic properties of the protein have been examined in detail [1,4]. Localization of these splits in the sequence of the α-subunit is therefore important for understanding the organisation of this protein in the membrane and the mechanism of cation translocation. Recently, the complete amino acid sequences of the α-subunit of $(Na^+ + K^+)$-ATPase from outer medulla of sheep [5] and pig [6] kidney and electroplax of

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Abbreviation: PTH, phenylthiohydantoin.

Torpedo californica [7] were deduced from nucleotide sequences, but the sites of selective protolytic cleavages have not been localized within the sequences.

To localize these sites, we isolated proteolytic fragments of the α-subunit from pig kidney by high resolution chromatography in $NaDodSO_4$. NH_2-terminal amino acid sequences of the fragments were determined in a gas phase sequenator and the specific cleavage sites were located within the sequences of the α-subunit. Structure function relationships are discussed on the basis of the information available about the consequences of these splits for the $(Na^+ + K^+)$-ATPase reaction and cation transport.

Methods

$(Na^+ + K^+)$-ATPase was purified in membrane-bound form from pig kidney outer medulla by incubation of crude membrane protein with $NaDodSO_4$ in presence of 3 mM ATP-Na_3 and centrifugation for 120 min at 48 000 rpm in zonal rotors as described before [8]. Incorporation of fluorescein isothiocyanate and titration of fluorescence responses were done as before [9,10]. Chymotryptic digestion and sequential cleavage by chymotrypsin and trypsin were also described before [4]. After cleavage the protein was solubilized with $NaDodSO_4$ and injected into a TSK 3000 SW column, 7.5 × 600 mm with 100 mm guard column. The system is equilibrated with 1% w/v $NaDodSO_4$, 100 mM sodium acetate, pH 4.5 at 20°C and operated at 0.5 ml/min by a Waters model 6000 A pump. Absorbance is monitored at 277 nm and fractions are collected at $\frac{1}{2}$ min intervals.

Amino acid sequences were determined using an Applied Biosystems Model 470A gas phase Protein Sequencer, essentially as described by Hewick et al. [11]. PTH amino acids were analyzed by reverse phase HPLC, using a Waters Nova-Pak column and the gradient elution system described in Waters Associates Applications Brief M3500. A Waters HPLC system including two M510 pumps, a WISP 710B autoinjector and a M440 dual channel absorbance detector was used. The detector was set to measure the sum of the absorbances at 254 nm for quantitative measurement of PTH amino acids and 313 nm for qualitative detection of breakdown products of PTH-Ser and PTH-Thr. The recovery of PTH amino acids at each cycle was measured with an integrative recorder (Waters M 730 Data Module). Yields of PTH-Ser and PTH-Thr were usually low and sometimes not detectable at all; but they could always be identified from the absorbance of breakdown peaks at 313 nm. PTH-Arg and PTH-His were also often recovered in low yields.

Results

The high resolution of the TSK 3000 SW column for separation of peptide fragments in $NaDodSO_4$ is illustrated by the data in Fig. 1. After elution with 1% $NaDodSO_4$ at pH 4.5 the α-subunit, β-subunit, 83 kDa fragment and 18

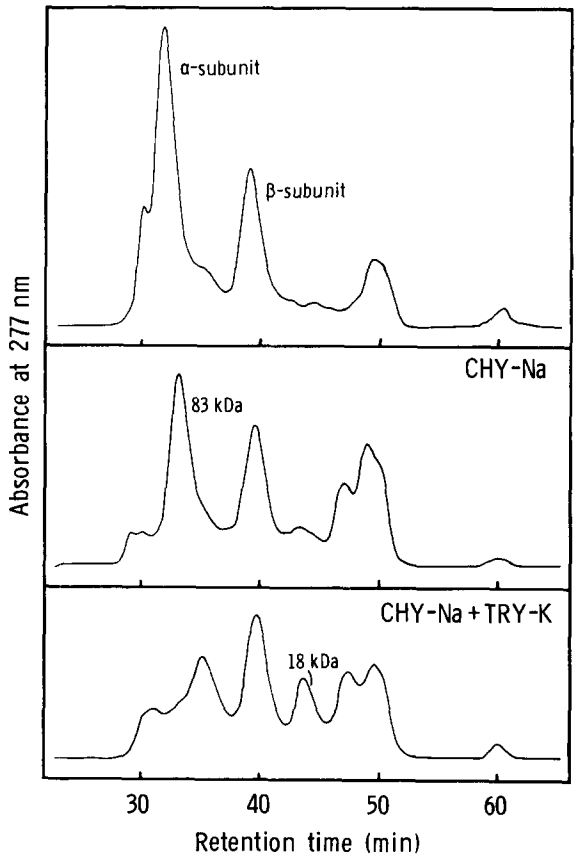

Fig. 1. Separation of α-subunit, β-subunit and proteolytic fragments by HPLC chromatography on TSK 3000 SW column equilibrated with 1% $NaDodSO_4$, 100 mM sodium acetate, pH 4.5 and eluted at 0.5 ml/min.

kDa fragment appear as separate peaks of peptides with well defined NH_2-terminal sequences. In contrast, isolation of the fragments arising by tryptic cleavage near the middle of the α-subunit poses problems. The two fragments are almost of equal size, 64 and 47 kDa. The COOH-terminal fragment of 64 kDa was eluted earlier than the β-subunit, while the smaller NH_2-terminal fragment could not be isolated. NH_2-terminal sequences are shown in Fig. 2 for the α-subunit. The NH_2-terminal 13 residues of the purified α-subunit of $(Na^+ + K^+)$-ATPase from pig kidney outer medulla are identical to the rat kidney sequence [21] and to the sequences deduced from nucleotide sequences of sheep [5] and pig [6] kidney. The sequences of α-subunit from sheep [5] and pig [6] kidney both consist of 1016 residues. They are identical except in 19 positions that are listed in Table I.

TABLE I

Differences in amino acid composition between sequences of α-subunit of sheep [5] and pig [6] kidney.

Residue in sheep sequence	Replacement in porcine sequence	Residue in sheep sequence	Replacement in porcine sequence
53-Asn	Ser	573-Val	Leu
58-Thr	Pro	654-Arg	Lys
102-Val	Ile	668-Pro	Ser
431-Asp	Glu	833-Gln	Lys
455-Val	Leu	841-Arg	Gln
468-Ala	Thr	866-Met	Leu
489-Ala	Pro	874-Asn	Ile
491-Ala	Thr	879-Ile	Leu
492-Gly	Ala	882-Thr	Asn
552-Met	Phe		

An extended sequencer run on the β-subunit identified residues 1–29 of the NH_2-terminus end of the protein (not shown). The sequence is identical to the sequence of β-subunit deduced from nucleotide sequences of pig [12] and sheep [13] kidney. The NH_2-terminus of the β-subunit is a highly polar sequence with 12 charged residues, that may form the antigenic site of β-subunit that has been identified at the cytoplasmic surface. The β-subunit has one transmembrane segment [12,13] and the bulk of the hydrophilic residues of the β-subunit are exposed at the extracellular surface [14].

Tryptic cleavage in NaCl medium

In NaCl medium (E_1Na) or after binding of ATP or ADP (E_1AXP) tryptic inactivation of $(Na^+ + K^+)$-ATPase and cleavage proceed in two phases [2]. The initial fast phase of inactivation is associated with release of small peptides from the NH_2-terminus of the α-subunit. This rapid and selective cleavage of one or more bonds near the NH_2-terminus alters the poise of equilibria between both dephosphoforms (E_1Na-E_2K) and phosphoforms (E_1P-E_2P) in the direction of the E_1-forms [15,16].

To localize this clip, tryptic cleavage of membrane bound $(Na^+ + K^+)$-ATPase was carried out in medium containing ligands (Na-ADP) that stabilize the E_1 conformation. NH_2-terminal sequencing of the protein eluting from the TSK 3000 SW column at the position of the α-subunit localizes the split to the Lys-30-Glu-31 bond in the sequence as shown in Fig. 2. Previously it was

				Na Try		
	1	10	20	30	40	50
Present data, Pig kidney α	NH_2-GRDKYEPAAV SEH			EVSMXXXKLS LXELXXKY		
Sheep kidney α	NH_2-GRDKYEPAAV SEHGDKKKAK KERDMDELKK			EVSMDDHKLS LDELHRKYGT		
Rat kidney α	NH_2-GRDKYEPAAV SEHG					
Rat axolemma α^+	NH_2-GREYSPAAEV AEVG					
Torpedo	NH_2-MGKGAASEKY QPAATSENAK NSKKSKSKTT DLDELKKEVS LDDHKLNLDE					
Brine shrimp	NH_2-AKGKQKKGKD LNELKKELDI DFHKIP					

Fig. 2. NH_2-terminal sequence of α-subunit and localization of fragment of α-subunit that was isolated after completion of the fast phase of tryptic cleavage in NaCl medium. Comparison with sequence data from sheep kidney [4], rat kidney and axolemma [21], *Torpedo californica* [7] and brine shrimp [24]

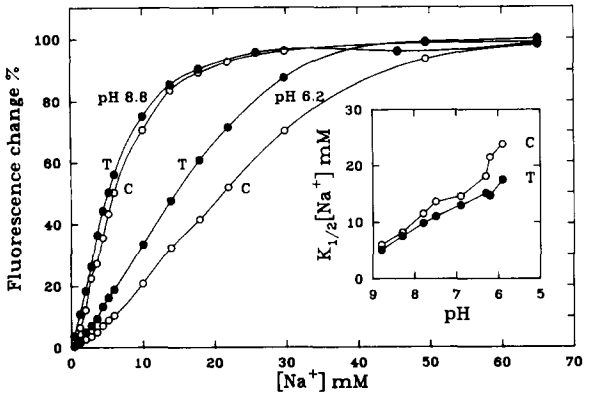

Fig. 3. Effect of tryptic cleavage between Lys-30 and Glu-31 on apparent affinity of $(Na^+ + K^+)$-ATPase in media of different pH. Fluorescein-$(Na^+ + K^+)$-ATPase, 20–25 μg protein, either control (C) or cleaved with tryptin (T) was titrated with Na^+ in presence of 1 mM KCl in 50 mM Tris-maleate with pH as indicated on the abscissa. NaCl was added from Hamilton syringes in portions of 0.5–1.0 μl. Fluorescence intensities were recorded at 519 nm with excitation wavelength 495 nm and 10 nm slits on both monochromators.

observed that an alanine formed the NH_2-terminal residue after this split [3] suggesting a split between Lys-18 and Ala-19. It is possible that trypsin may cleave at several positions at the NH_2-terminus, and that cleavage at bond 30–31 represents a more extensive degradation when cleavage is carried to completion for preparation of the 'invalid' $(Na^+ + K^+)$-ATPase.

The experiment in Fig. 3 shows the effect of this cleavage on transition from E_2K to E_1Na at different values of pH. Control (C) or cleaved (T) preparations of membrane-bound $(Na^+ + K^+)$-ATPase incorporated identical amounts of fluorescein isothiocyanate covalently. Titration of the fluorescence responses with NaCl showed that $K_{1/2}$(Na) was decreased when pH was raised. At high pH (7–8.8), $K_{1/2}$(Na) was only slightly higher for control that for cleaved preparations. At low pH (5.9–6.2) a larger difference in $K_{1/2}$(Na) became apparent. In the range of pH values from 5.9 to 6.3 $K_{1/2}$ of the cleaved preparation (T) was significantly lower than for control (C). Removal of 30 residues of the NH_2-terminal end of the α-subunit thus increased the apparent affinity of the enzyme for Na^+. This result suggests that removal of an ionizable group with pK near 6, e.g. His-13, is responsible for the increase in affinity for Na^+.

Chymotryptic cleavage in NaCl at low ionic strength

A bond located in the N-terminal half of the α-subunit is cleaved specifically by chymotrypsin when the α-subunit is in the E_1-form [1,4]. As demonstrated in Fig. 1 the large fragment remaining in the membrane could be purified after completion of chymotryptic cleavage because secondary cleavage is negligible. Sequence data in Fig. 4 show that the 13 residues forming the NH_2-terminus of this fragment are identical to a sequence starting at Ala-267 of the α-subunit from sheep [5] or pig [6] kidney outer medulla. Thus, chymotrypsin cleaves between Leu-266 and Ala-267. As calculated from the sequence, the molecular weight of the fragment (Residue 267–1016) is M_r 82978. This compositional value is greater than the mass of 78 kDa determined by polyacrylamide gel electrophoresis in $NaDodSO_4$ [1,4].

The molecular mass of the fragment arising after cleavage with trypsin in NaCl medium (previously designated the 78 kDa fragment) appears by polyacrylamide gel electrophoresis in $NaDodSO_4$ to be identical to the fragment arising after chymotryptic cleavage. The tryptic fragment could not be isolated with the present techniques. Previously, the NH_2-terminus of this fragment was identified as Ile [3]. It is therefore most likely that tryptic cleavage of the E_1-form occurs be-

	240	250	260	270	280
			Na		
			Try Chy		
Sheep kidney α-subunit	TNCVEGTARG	IVVYTGDRTV	MGRIATLASG	LEGGQTPIAA	EIEHFIHIIT
Present data, fragment M_r 82978			ASG	LEGGQTPIAA	E
Present data, fragment M_r 18722			ASG	LEGGQTPIAA	

Fig. 4. NH_2-terminal sequence of fragment with M_r 82978 after chymotryptic cleavage in NaCl and fragment with M_r 18722 after sequential tryptic and chymotryptic cleavage.

tween Arg-262 and Ile-263 as indicated in Fig. 4.

Sequential cleavage first with chymotrypsin in NaCl and then with trypsin in KCl media allows isolation (Fig. 1) of a fragment with estimated size of 18 kDa that incorporates ^{32}P from [γ-^{32}P]ATP [1,4]. Sequence data in Fig. 4 shows that the NH_2-terminus of this fragment is identical to that of the large fragment arising after chymotryptic cleavage in NaCl medium. The fragment is therefore generated by removing the NH_2-terminal 171 residues (Residue 267–438) from the fragment with M_r 82978 (see below). From the sequence this fragment has M_r 18722 in good agreement with the molecular mass of 18 kDa that was determined by polyacrylamide gel electrophoresis in $NaDodSO_4$ [1,4].

After sequential cleavage, a 64 kDa fragment arises by tryptic cleavage of the 83 kDa fragment. In Fig. 1 it is seen that this 64 kDa fragment was eluted earlier than the β-subunit (Fig. 1). It had NH_2-terminus consisting of Ala-Val-Ala-Gly-X-Ala-X-Glu-X-Ala-Leu-Leu thus identifying the cleavage between Arg-438 and Ala-439. This gives M_r 64274 for the COOH-terminal fragment (residue 439–1016) that was previously designated the 58 kDa fragment. Data of NH_2-termini and molecular weights of fragments are summarized in Fig. 5. The NH_2-terminal fragments arising after tryptic cleavage in KCl medium have M_r 47998 (Residue 1–438) or M_r 44528 (Residue 31–438), respectively, as compared with the molecular masses 46 kDa and 41 kDa that were determined by gel electrophoresis in $NaDodSO_4$ [1,2]. Comparison of compositional values with molecular masses determined by gel-electrophoresis in $NaDodSO_4$ thus shows a good correspondence for the NH_2-terminal half of the α-subunit, while the molecular masses of the COOH-terminal half were underestimated by gel electrophoresis, like the molecular mass of the entire α-subunit. Relatively fast migration in gel electrophoresis with underestimation of the molecular mass of the α-subunit may thus be related to abnormal binding of $NaDodSO_4$ or incomplete unfolding of segments within the COOH-terminal half of the α-subunit.

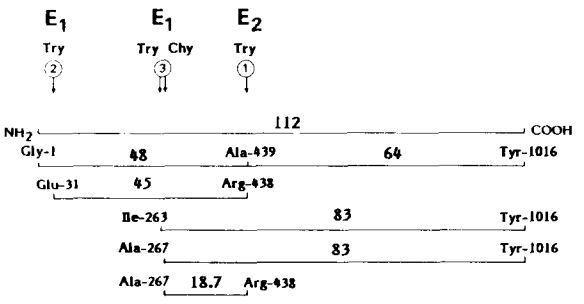

Fig. 5. Linear model of α-subunit (1016 residues, M_r 112254) or ($Na^+ + K^+$)-ATPase from pig kidney with position of tryptic and chymotryptic splits in E_1-forms and in E_2 forms of the protein. Molecular weights of the fragments are calculated from the aminoacid composition of the fragments in the sequences determined for α-subunit from pig [6] kidney.

Discussion

Chymotryptic cleavage of bond 3 and tryptic cleavage of bond 1

The site of primary chymotryptic clevage at low ionic strength in NaCl medium (E_1-form) of the α-subunit is identified as the bond between Leu-266 and Ala-267. The data identifies the fragment with M_r 82978 arising after primary chymotryptic cleavage and the fragment with M_r 18722 arising after sequential cleavage with chymotrypsin and trypsin. This confirms the localization of the chymotryptic split to the NH_2-terminal half of the α-subunit that was based on experiments with phosphorylation of the fragments [4]. The fragment with M_r 18722 incorporates phosphate from [γ-^{32}P]ATP into the phosphorylation site, Asp-369. It also incorporates hydrophobic labels from the lipid bilayer and it participates in formation of the binding site for ouabain at the extracellular surface. This information together with the localization of the cleavage points at the cytoplasmic surface [17] were essential features for the model of the path of the α-subunit in the membrane bilayer [1,18].

This chymotryptic split interferes with motion of segments in the α-subunit that are essential for E_1-E_2 transition [4]. Binding sites for cations and nucleotides are preserved after cleavage of the bond between Leu-266 and Ala-267, but cation exchange and conformational transitions in the protein are abolished. In contrast, tryptic cleavage between Arg-438 and Ala-439 does not interfere with E_1-E_2 transitions and cation exchange [1,4]. Based on these data a model for the structural

transition in α-subunit was proposed in which E_1-E_2 transition involves motion of a part of the 18 kDa fragment from relatively hydrophilic to a more hydrophobic environment.

Function of the NH_2-terminal part of the α-subunit

The experiments with cleavage of the bond between Lys-30 and Glu-31 illustrates the role of the NH_2-terminal segment for the E_1-E_2 transition. Tryptic cleavage of this bond and release of the NH_2-terminal 30 residues increases the apparent affinity for Na^+ at low pH. Studies of phosphorylation kinetics [15] and conformational transitions after this split [16] showed that the 50–60% reduction in $(Na^+ + K^+)$-ATPase activity and Na^+,K^+-transport can be ascribed to a change in poise of E_1-E_2 equilbrium in the direction of E_1. This applies both to dephospho- and phospho-forms of the protein. Since removal of 30 NH_2-terminal residues is the only modification of the cleaved 'invalid $(Na^+ + K^+)$-ATPase' enzyme, the data allow the conclusion that charged residues in this segment are important for E_1-E_2 transitions. The dependence of $K_{1/2}(Na)$ on pH for both control and cleaved enzyme shows that H^+ competes with Na^+ for binding to anionic sites as the deprotonated form has higher affinity for Na^+ than the protonated form, in agreement with observations on $(Na^+ + K^+)$-ATPase from shark rectal gland [19]. Comparison of control and cleaved $(Na^+ + K^+)$-ATPase shows that protonation of an ionizable group with pK 5.9–6.3 causes a decrease in apparent affinity for Na^+ since removal of the NH_2-terminal segment increases the apparent affinity for Na^+ at low pH. This increase in apparent affinity for Na^+ following an increase in pH can be compared with the Bohr effect, i.e., an increase in pH increases the affinity of hemoglobin for oxygen [20].

In renal $(Na^+ + K^+)$-ATPase, the ionizable group responsible for these effects is likely to be His-13, since the weakly ionized imidazole group has a pK near 6, while pK values are much higher for the positively charged groups of lysine and arginine that are abundant in the NH_2-terminal segment. As the difference in $K_{1/2}(Na)$ is apparent only at low pH, protonation of this group antagonizes Na^+ binding. Removal of this group causes a shift in E_1-E_2 equilibrium in direction of E_1 forms. Protonation of the His-13 residue therefore facilitates transition from E_1- to E_2-forms of the protein.

It is interesting to note that His-13 in the NH_2-terminal sequence of the α-subunit from kidney is replaced with Val-13 in α^+-subunit from axolema of rats [21]. Kinetic analysis of $(Na^+ + K^+)$-ATPase containing α or α^+ shows cooperative differences with different affinities for ouabain reflecting different rates of conformational transition during enzyme turnover [22]. This agrees with the present observation, but the mechanism behind the influence of the NH_2 terminal segment on E_1-E_2 transition remains to be established. The presence of excess positive charges in this segment (8 lysines and 2 arginines among 30 residues) suggests involvement in salt bridge formation. Models involving formation and dissociation of salt bridges as part of translocation mechanisms for Na^+ and K^+ have been examined before [23]. The NH_2-terminal segment may engage in salt bridge formation with other cytoplasmic domains within the same α-subunit. It is in agreement with such a role of salt bridge formation that the exposure of bond 30-31 to trypsinolysis depends strongly on ionic strength [2].

The particular features of the NH_2-terminus of α-subunit from mammalian kidney are not conserved in α-subunit from *Torpedo* [7] and brine shrimp [24] (Cf. Fig. 2) or in Ca^{2+}-ATPase from sarcoplasmic reticulum [25]. Tryptic cleavage patterns and effects of selective cleavage on $(Na^+ + K^+)$-ATPase activity and Na^+,K^+-transport have not been examined in preparations from *Torpedo*, brine shrimp or rectal gland. Cleavage of the NH_2-terminus does not occur in Ca^{2+}-ATPase [26]. It is possible that a regulatory function of the NH_2-terminal part of the α-subunit in E_1-E_2 transitions is a unique feature of α-subunit in $(Na^+ + K^+)$-ATPase from mammalian kidney that may be related to the regulation of Na^+/K^+-pumping in renal medulla.

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