Structure of the extra-membranous domain of the β-subunit of (Na,K)-ATPase revealed by the sequences of its tryptic peptides

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Membrane bound dog kidney (Na,K)-ATPase was digested with trypsin. The peptides that were recovered in the supernatant were purified and sequenced. By comparing these results with the sequence of α- and human β-subunits, the location of each of the peptides could be allotted. Both accessibility to trypsin and the facility of release into the water phase indicated that these peptides were derived from the exposed surface of the intact enzyme. The sequence, GXGXXG, reported in the *Torpedo californica* β-subunit [(1986) FEBS Lett. 196, 315–319] was likely a mere coincidence with the sequence of the dinucleotide-binding site, since the last glycine was replaced by proline in the sequence of the dog β-subunit. A disulfide bridge was found within a peptide derived from the β-subunit. A possible model for the β-subunit structure is proposed.

(Na^+,K^+)-ATPase *Amino acid sequence* *Trypsin digestion* *Nucleotide-binding site* *Disulfide bridge*

1. INTRODUCTION

The ion transport (Na,K)-ATPase is an intrinsic protein that spans across the eukaryotic cell membrane. The enzyme consists of two subunits, a larger catalytic subunit (α) with an M_r of about 112000 and a smaller glycoprotein subunit (β) with an M_r of about 35000 [1,2]. The amino acid sequences of both subunits have been deduced by means of cDNA sequencing from the enzymes obtained from different sources: electric ray (*Torpedo californica*) [3] and sheep [4] for the α-subunit and *Torpedo* [5] and human [6] for the β-subunit. The deduced sequences have predicted the transmembrane topologies of the two subunits, where the α- and β-subunit span the membrane 6–8 times and only once, respectively. A large portion of each subunit is exposed to the hydrophilic surfaces of the membrane. As far as the α-subunit is concerned, this transmembrane topology has been confirmed by the isolation of the chemically labeled specific peptides from various regions [7–9].

Regarding the β-subunit, the analyses of the specific peptides have not been done since the functions of the β-subunit are still unclear. We, however, found the amino acid sequence GXGXXG, which is characteristic of the dinucleotide-binding site [10] in the deduced sequence of the *Torpedo* β-subunit. If the sequence really functions as the binding site, the peptide containing the sequence should be, at least, exposed to the aqueous phase and hence proteolytic cleavage of the membrane-bound (Na,K)-ATPase is expected to release the peptide from the membrane.

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Here, we have sequenced some of the water-soluble tryptic peptides released from the membrane-bound (Na,K)-ATPase of dog kidney. Among them, the dinucleotide-binding peptide was identified, but the sequence was GXGXXP (where X can be any amino acid).

2. MATERIALS AND METHODS

(Na,K)-ATPase from the outer medulla of dog kidneys was purified; these preparations had specific activities of approx. 1500 μmol P_i/mg protein per h [11]. The native membrane-bound enzyme (2 mg) was digested with TPCK-trypsin (40 μg) in a medium consisting of 50 mM imidazole/HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 1 mM $CaCl_2$, at 37°C. TPCK-trypsin was added twice at a 1.5 h interval. The digestion was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. The digest was centrifuged at 160000 × g for 1 h, and the supernatant containing the soluble peptides was applied on a reverse-phase C-4 (Bio-Rad, Hi-pore) column equilibrated in 0.1% trifluoroacetic acid (TFA). Elution was carried out with a linear gradient of 10–40% acetonitrile containing 0.1% TFA. Each fraction was further purified by a second run of HPLC on the same column with a less steep gradient of acetonitrile. The purified peptides were sequenced on a gas-phase sequencer (Applied Biosystems, model 470A-120A).

3. RESULTS AND DISCUSSION

When the membrane-bound (Na,K)-ATPase of dog kidney was digested with trypsin, many peptides were released into the aqueous phase as shown in fig.1. These peptides were most likely derived from the exposed domains of the enzyme. We then purified them to homogeneity by second runs on HPLC and sequenced as many of these peptides as possible, and we searched for the homologous sequence to each peptide in the deduced primary structures of sheep α-subunit [4] as well as human β-subunit [6]. The results are shown in table 1. Eleven tryptic peptides, 5 in the α- and 6 in the β-subunits, were allotted with a homology of more than 70%. Other peptides in fig.1 could not be purified or yielded, even if purified, sequences with homologies of less than 70%. The

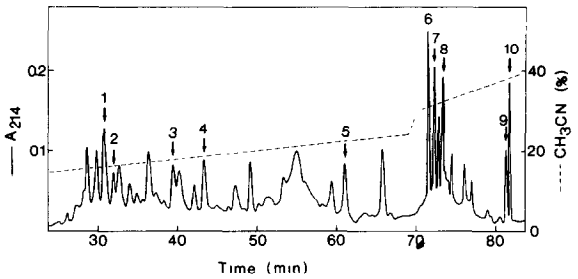

Fig.1. Reverse-phase HPLC of the water-soluble tryptic peptides released from dog kidney (Na,K)-ATPase. The tryptic peptides in the supernatant were applied to a Bio-Rad C-4 column (4.6 × 259 mm) equilibrated with 0.1% TFA and eluted with linear gradients of 95% acetonitrile containing 0.1% TFA. Absorbance at 214 nm was monitored.

Table 1

Sequences of the peptides released into the supernatant by trypsin digestion of dog kidney (Na,K)-ATPase

Peptide no.	Subunit	Sequence
1	β	86-TLISFKPNDPK
2	α	496-HLLVMK
3	β	76-GLEIVPQIQK
4	α	626-IISEGNETVEDIAAR
5	β	112-YKDSAQKDDMIF---
6	α	887-WINDVLDSYGQQWQYEQR
7(1)	β	151-FKLEWLGXXSGIND---
7(2)	β	171-EGKPXVLIK
8	α	379-MTVAHMWFDN---
9	β	224-FGNVEYFGLGWYPGF---
10	α	520-EQPLDEELKDAFQ---

The number at the top of each sequence indicates the position of the first amino acid of the respective peptide in the whole sequences of the α- and β-subunit of sheep and human enzyme, respectively. Peptide numbers correspond to peak numbers in fig.1. X, unidentified amino acids

significance of the tryptic, water-soluble peptides belonging to the α-subunit has been reported [9], and the sequences of the peptides in the *Torpedo* α-subunit were confirmed in the dog α-subunit. Therefore, we will mainly discuss here the peptides derived from the β-subunit.

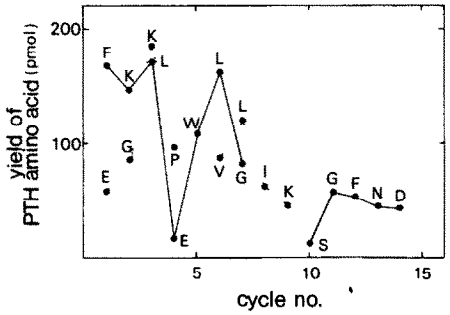

Fig.2. Recovery of PTH-amino acids in sequence analysis of peptide-7. See text for details.

The sequence GXGXXG, which is characteristic of the dinucleotide-binding region of various dinucleotide-binding proteins [10], has been found in the sequence of the *Torpedo* β-subunit. Peptide-9 contained a similar sequence with the exception that the last glycine was replaced by proline as in the case of the β-subunit of HeLa cells. Although some have claimed that it is possible that the replacement is specific to the enzyme of transformed cells such as HeLa cells and not a general feature of the human enzyme [6], the results described herein suggest that the sequence recognized in the *Torpedo* enzyme only coincidentally resembles that of the dinucleotide-binding region.

The sequence of peptide-7 provided information about the part of the β-subunit exposed to the outside of the membrane. Two kinds of PTH-amino acid were recovered in each cycle up to the 7th cycle except the 5th one. The next 7 cycles yielded a single PTH-amino acid in each cycle in a sufficient quantity (fig.2). These results suggest that peptide-7 was composed of two kinds of peptides. The sequence of the last 5 amino acids determined, SGIND, was recognized in the primary structure of the human β-subunit (S(160)–D(164)) except that I was replaced by L(162). The sequence of the human β-subunit around the position is R(150)FKLGWLGNCSGLNDETYGYK(170). Amino acids from F(151) to G(157) corresponded to the PTH-amino acids from the 1st to the 7th cycle in fig.2 in that order. A highly homologous sequence corresponding to another sequence in peptide-7 was recognized in the human β-subunit (E(171)–K(179)). From these results, we could determine the sequences of the two peptides that compose peptide-7, which are shown in table 1.

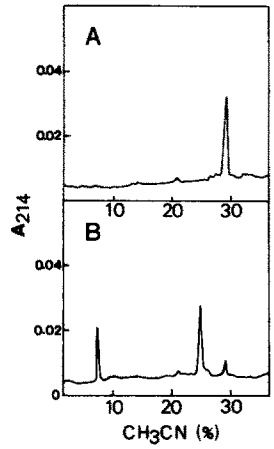

Fig.3. HPLC elution patterns of peptide-7 with (B) or without (A) the reduction with 5 mM dithiothreitol containing 50 mM imidazole/HCl (pH 7.5) at 50°C for 4 h.

There are three unidentified amino acids in the sequences of peptide-7: the 8th and 9th residues in peptide-7(1) and the 5th residue in peptide-7(2). By comparing the sequences of peptide-7 with the sequence of the human β-subunit, it is likely that the 8th amino acid of peptide-7(1) is asparagine and the other two unknown ones are cysteine. The asparagine residue in the sequence of NXS (where X can be any amino acid) is well-known as the possible glycosylation site. The 8th asparagine residue in peptide-7(1) in NXS, which is probably glycosylated, might be the reason why only one PTH-amino acid (isoleucine) was recovered in the 8th cycle of the Edman degradation of peptide-7.

The two peptides of peptide-7 behaved like a single peptide throughout the purification procedures. This can be explained if the two putative cysteines in peptide-7 were cross-linked by a disulfide bridge. To determine if this was the case, peptide-7 was prepared from the enzyme whose free cysteine residues had been modified with 5 mM *N*-ethylmaleimide to block spontaneous formation of disulfides among the cysteines. The peptide-7 thus prepared was eluted from the HPLC column at the acetonitrile concentration of approx. 30% (fig.3A). However, when the peptide was reduced with 5 mM dithiothreitol and applied on the column, two additional peaks appeared in the HPLC pattern. The original peak decreased substantially as shown in fig.3. These results

strongly suggested that peptide-7 was composed of two kinds of peptides cross-linked to each other by a disulfide bridge. The number of disulfide bridges in a molecule of (Na,K)-ATPase has been reported to be at most two, both of which are on the β-subunit [12,13]. One disulfide bridge which is essential for the activity of the enzyme is highly resistant to the reducing agent [12,14]. We have not yet obtained any evidence to enable us to discuss whether the disulfide bridge in peptide-7 may or not be the essential one. To answer this question, specific chemical labeling studies are now in progress in our laboratory.

Peptide-3 was homologous to the sequence from G(76) to K(85) of the human β-subunit. Although glycine-75 is preceded by two prolines in the human sequence, the latter proline may be replaced by lysine or arginine in the sequence of the dog β-subunit.

Peptide-1 and peptide-5 were homologous to the sequences from T(86) to K(96) and from Y(112) to F(123) of the human β-subunit, respectively.

Based on the hydropathic profile of the β-subunit together with the results reported in this article, a possible model of the (Na,K)-ATPase β-subunit structure is proposed and is shown in fig.4. Chin [15] has also proposed a similar structure from the results of papain fragmentation of the β-subunit. However, it differs from ours in a few points. The crucial difference is that he has postulated the existence of a second transmembrane segment in the carboxy-terminal portion in order to associate the carboxy-terminal piece with the lipid bilayer. Consequently, the carboxy-terminus is at the cytoplasmic side in his model. However, if the site of papain hydrolysis in his experiment is located between the cysteine residues at 159 and 175, the carboxy-terminal piece can be associated with the membrane via the disulfide bridge formed between the two cysteines.

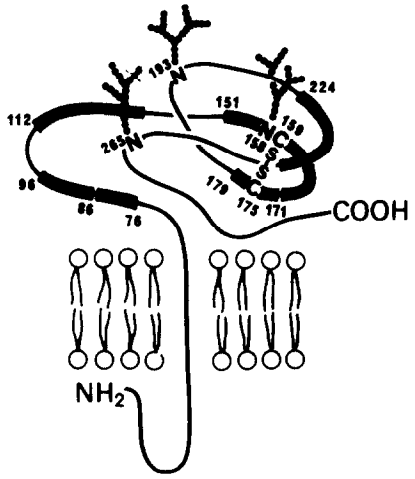

Fig.4. The locations of water-soluble tryptic peptides of the dog kidney (Na,K)-ATPase β-subunit. The locations are shown on the amino acid sequence of the human β-subunit. Three possible glycosylation sites and the position of the disulfide bridge are drawn.

In the sequences determined in this report, we can find lysine followed by aspartic acid (peptide-5, and -10) or by leucine (peptide-7(1)). Side chains with a net negative charge which are adjacent to a lysyl residue tend to reduce significantly the rate of hydrolysis [16]. This may be the reason why lysyl-aspartic acid has not been cleaved by trypsin in this experiment. We cannot find the reason why lysyl-leucine in peptide-7 is resistant to trypsin digestion, but the disulfide bridge within peptide-7 may interfere with the digestion by trypsin.

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