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Neutral Endopeptidase Neprilysin Is Copurified with Na,K-ATPase from Rabbit Outer Medulla and Hydrolyzes Its α-Subunit

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Abstract—Preparations of Na,K-ATPase from outer medulla of rabbit kidney purified in accordance with the method of P. L. Jorgensen were shown to contain as admixture a protease that moves with α-subunit (~100 kDa) as a single protein band during one-dimensional SDS-PAGE. The electro-elution of proteins of this band from polyacrylamide gel results in the appearance of two protein fragments (~67 and 55 kDa) that are stained with polyclonal antibodies against Na,K-ATPase α-subunit. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis showed that the neutral membrane-bound endopeptidase neprilysin is located in one protein band together with the Na,K-ATPase α-subunit. Addition of thiorphan, a specific inhibitor of neutral endopeptidase, eliminates proteolysis of the α-subunit. The data demonstrate that Na,K-ATPase α-subunit may be a natural target for neprilysin.

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Key words: Na,K-ATPase α-subunit, neprilysin, proteolysis, mammalian kidney

Na,K-ATPase (EC 3.6.1.3) is a membrane protein complex that actively transports Na^+ and K^+ across the plasma membrane against the electrochemical gradient. The enzyme consists of two different subunits. Its catalytic α-subunit (~100 kDa) forms a transmembrane channel and couples ATP hydrolysis with the movement of Na^+ and K^+ against their electrochemical gradients. Its smaller β-subunit (~35 kDa, ~55 kDa after glycosylation) is tightly bound to the catalytic one and has mainly regulatory function, in particular, it is involved in the delivery of the whole complex to the plasma membrane after its synthesis [1]. The third subunit (~7 kDa) that can be represented by different proteins from the FXYD protein family is also found in preparations of Na,K-ATPase from various tissues. It also plays a mainly regulatory role [1].

A method for Na,K-ATPase purification from mammalian kidney was developed in the 1970s by Jorgensen [2]. According to this method microsomal fraction enriched in Na,K-ATPase activity is obtained from kidney outer medulla using differential centrifugation. Then the microsomes are treated with SDS, which extracts various proteins, whereas Na,K-ATPase remains embedded in the membrane. Subsequent centrifugation in a sucrose density gradient produces a pellet consisting of membrane sheets including mainly Na,K-ATPase α- and β-subunits.

Although the method is widely used in different laboratories, a problem of its reproducibility still exists. Sometimes, besides α- and β-subunits, small amounts of other proteins are found in the preparations of Na,K-ATPase from mammalian kidney. Among them there are several proteins with molecular masses less than 100 kDa that might be the products of α-subunit proteolysis. The goal of this study was to reveal proteins with protease activity in such preparations of Na,K-ATPase.

MATERIALS AND METHODS

ATP, SDS, EDTA, glycine, imidazole, sucrose, thiorphan, and Tris were from Sigma (USA) and Khelikon (Russia); protease inhibitor cocktail including AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride),

Abbreviations: LC/MS/MS, liquid chromatography/tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis.
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aprotinin, bestatin, E4 (L-transepoxy-succinyl-leucyl-amido(4-guanidino)butan), and leupeptin was purchased from Amresco (USA); acrylamide, methylene-bis-acrylamide, and other reagents for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad (USA). Polyclonal mouse antibodies against Na,K-ATPase α-subunit were obtained in our laboratory as described early [3] using as antigen the α1-subunit from rabbit kidney that was obtained by electrophoretic elution from polyacrylamide gel. Only preparations of α-subunit that did not demonstrate proteolysis during the electro-elution procedure were used for immunization. All other reagents of the highest purity available were produced in Russia.

Na,K-ATPase was purified from rabbit kidney in accordance with the method described by Jorgensen [2]. In some cases protease inhibitor cocktail, EDTA (5 mM), and thiorphan (5 μM) were added to the buffers for purification. Purity of final preparations was determined using the Laemmli procedure of SDS-PAGE [4] with 3.5% stacking and 5-20% gradient running gel. After electrophoresis, proteins were stained with Coomassie Brilliant Blue. Protein concentration was measured according to the method of Lowry et al. [5].

Na,K-ATPase α-subunit was obtained by an electro-elution procedure. SDS-PAGE of Na,K-ATPase preparations was conducted as described above using gel with the thickness of 2 mm. Aliquots containing 15 μg of protein were loaded onto each lane. After electrophoresis, two strips were cut off the two sides of the gel and stained with Coomassie Brilliant Blue for the identification of the α-subunit. The corresponding region of unstained gel was separated and cut into small pieces, which were put in a chamber for electro-elution (Model 422 Electro-Eluter; Bio-Rad). Electro-elution was performed for 3 h with a current 10 mA per tube. Then purity of the α-subunit was determined by SDS-PAGE.

Western blot analysis was performed as described earlier [3].

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis of proteins in the band corresponding to Na,K-ATPase α-subunit was done in the Center of Proteomic Studies, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Moscow. Data were analyzed using the Data Analyses 2.1 program (Bruker Daltonics), and the search in the database of translated sequences of nucleic acids NCBI was preformed using the Mascot program (Matrix Science).

RESULTS AND DISCUSSION

Analysis of Na,K-ATPase preparations purified from rabbit kidney according to the method of Jorgensen using SDS-PAGE revealed that small amounts of proteins with molecular masses 78, 70, 67, 37, and 32 kDa (Fig. 1, lane *2*) often may be found in the preparations. Some of them were not detected in microsomal fraction that was used as intermediate product in the procedure of purification (Fig. 1, lanes *1* and *2*). We suggested that some of these proteins may be fragments produced by proteolytic degradation of Na,K-ATPase subunits that occurs during the last steps of purification (treatment of microsomes by SDS or subsequent centrifugation). In fact, the amounts of such proteins in preparations were decreased if commercial cocktail of protease inhibitors was added to the buffers used in all steps of purification. Cocktail used in our experiments includes inhibitors of serine, cysteine, and lysosomal proteases as well as inhibitor of aminopeptidase. However some proteins, in particular, 67-kDa protein, were still sometimes found in the final preparation even if the purification procedure was performed in the presence of the protease inhibitor cocktail.

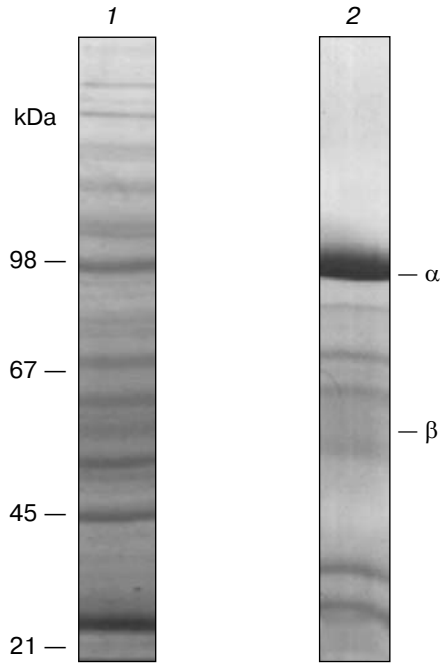

Fig. 1. Proteins of microsomal fraction from rabbit renal medulla (*1*) and preparation of purified Na,K-ATPase (*2*) after their separation by SDS-PAGE. Fifteen micrograms of protein was loaded on each lane.

Experiments with purification of Na,K-ATPase α-subunit by electro-elution showed independently that, besides the α-subunit, proteins of 67- and 55-kDa may also be found in the preparations (Fig. 2b). Because gel particles for electro-elution were taken from the region where Na,K-ATPase α-subunit or some other proteins with close molecular mass are located, we suggested that preparations of Na,K-ATPase may contain a protease with electrophoretic mobility corresponding to 100 kDa protein. The protease can cleave Na,K-ATPase α-subunit into two smaller fragments. Indeed, both protein fragments obtained during the procedure of electro-elution as

well as the α-subunit itself were stained by polyclonal antibodies against the α1-subunit. Figure 2c demonstrates result of Western-blot analysis of Na,K-ATPase α-subunit preparation in which almost complete proteolysis of this protein occurred.

To inhibit metalloprotease activity, we also added 5 mM EDTA to buffers for electro-elution and for storage of α-subunit. However, addition of EDTA only slowed the process of cleavage but did not inhibit it completely.

We tried to identify what proteins are located in the protein band corresponding to Na,K-ATPase α-subunit using LC/MS/MS.

The analysis revealed six peptides, amino acid sequences of which were characterized by high probability of nonrandom coincidence with amino acid sequences of specific proteins.

Sequences of five peptides found coincide with the sequences of certain fragments of Na,K-ATPase α1-subunit chain from different species including rabbit. This may be explained by high homology of amino acid sequences of α1-subunit in different species [6]. The sixth peptide originated from the protease neprilysin.

Neprilysin (EC 3.4.24.11) is a neutral zinc metalloendopeptidase that is an integral membrane protein found in the plasma membrane of many cell types [7]. Thiorphan (DL-3-mercapto-2-benzylpropranoyl)-glycine) is a specific inhibitor of neutral endopeptidases including neprilysin [8]. In our experiments the addition of thiorphan to the buffers used for purification of Na,K-ATPase and separation of its α-subunit by electro-elution prevented the proteolysis of this protein (data not shown).

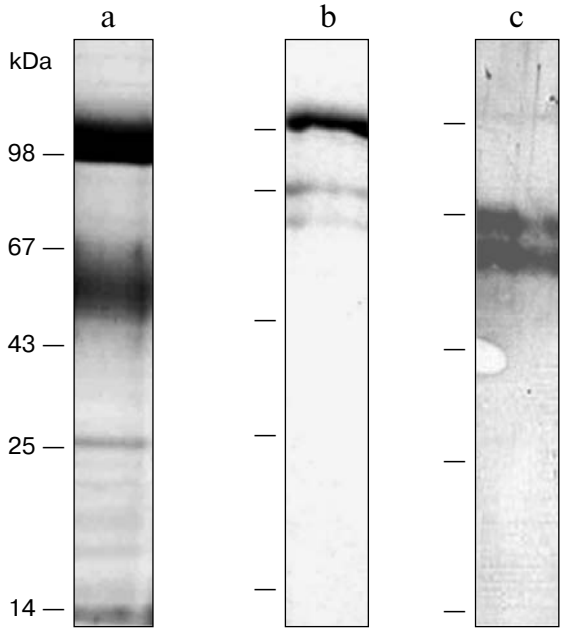

Fig. 2. SDS-PAGE of proteins of purified Na,K-ATPase (a) and its α-subunit (b) obtained by electro-elution from gel (a). c) Western-blot analysis of α-subunit obtained by electro-elution. The gel was stained using polyclonal antibodies against Na,K-ATPase α-subunit.

The data obtained in our study demonstrate that: 1) preparations of Na,K-ATPase from rabbit renal medulla may contain as admixture neutral endopeptidase neprilysin having the same electrophoretic mobility as Na,K-ATPase α-subunit; 2) Na,K-ATPase α-subunit (at least in the presence of SDS) may be cleaved by this protease into two fragments. The presence of neprilysin in preparations of Na,K-ATPase is confirmed by the LC/MS/MS analysis and by the suppression of α-subunit cleavage by specific inhibitor thiorphan.

Neprilysin was shown to be Zn-dependent protease. Nevertheless, in our experiments proteolytic activity was not completely inhibited in the presence of 5 mM of the divalent metal ion chelator EDTA. However, it is in agreement with earlier study that showed that neutral Zn-dependent endopeptidase from brush border membrane of rabbit kidney is able to preserve about 10% of its activity in the presence of this concentration of EDTA [9].

Because the molecular mass of neprilysin is within the range of 90-110 kDa [7], which is close to the molecular mass of Na,K-ATPase α-subunit, this protein cannot be revealed in preparations of Na,K-ATPase using one-dimensional SDS-PAGE: it moves in polyacrylamide gel together with α-subunit in a single protein band. Being masked in this way, neprilysin may be responsible for the appearance of some protein impurities (at least, 67- and 55-kDa protein bands) in preparations of Na,K-ATPase.

The precise function of neprilysin *in vivo* remains unknown, but it is suggested that this protease participates in the regulation of metabolism of a number specific neuropeptides and signaling peptides, for example, enkephalins [10] and atrial natriuretic peptide [11]. Neprilysin is also a major amyloid-beta (Abeta) degrading enzyme in brain and has been implicated in the pathogenesis of Alzheimer's disease [12]. In kidney it may play a special role in the degradation of peptides and proteins, and this function was shown to localize in proximal convoluted tubule [9].

The presence of neprilysin in purified preparations of Na,K-ATPase obtained from renal medulla may be indication of the contamination of medulla by traces of cortical layer that can take place during the procedure of medulla separation. But on the other hand, our data demonstrate that α-subunit can be cleaved by neprilysin into two fragments (Fig. 2). We did not observe further cleavage of these two fragments during longer storage of proteins eluted from the band where α-subunit and neprilysin are located. This suggests that there is a specific cleavage site for neprilysin in the polypeptide chain of the Na,K-ATPase α-subunit. However, the splitting of α-subunit in our experiments was observed mainly in the presence of SDS when it does not adopt its native confor-

mation. So we cannot contend now that this process can be physiologically relevant. Moreover, if neprilysin cleaves α-subunit into two fragments with indicated molecular masses, the site of cleavage should be located in the middle of the polypeptide chain that is the cytoplasmic domain. However, the neprilysin active site is exposed to the extracellular space.

But suggesting that this cleavage may occur *in vivo*, we can speculate that the site of cleavage of Na,K-ATPase α-subunit by neprilysin may be located in the loops exposed outside the cell. Taking into account that the main requirement neprilysin specificity is a presence of hydrophobic amino acid (often phenylalanine) on the C-terminal side of the cleaving bond and the molecular masses of proteolytic fragments obtained, we propose that neprilysin cleaves the polypeptide chain of α-subunit between transmembrane domains M3 and M4 or M5 and M6. But in these cases the proteolytic fragments should have molecular masses about 34 and 80 kDa or 20 and 94 kDa. However, one cannot exclude that a small fragment was cleaved by neprilysin to small peptides and the largest fragment was cleaved yielding fragments with molecular masses about 67 and 55 kDa. Further studies should be carried out to answer the question: is Na,K-ATPase α-subunit a natural target for neprilysin or not?

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