Structural analysis of the products of chymotryptic cleavage of the E1 form of Na,K-ATPase α-subunit: identification of the N-terminal fragments containing the transmembrane H_1-H_2 domain

Alexander Ivanov, Amir Askari, Nikolai N. Modyanov*

Department of Pharmacology, Medical College of Ohio, Toledo, OH 43614, USA

Received 10 November 1997

Abstract Chymotryptic cleavage of the Na,K-ATPase in NaCl medium abolishes ATPase activity and alters other functional parameters. The structure of this modified enzyme is uncertain since only one product of selective proteolysis, the 83-kDa fragment of the α-subunit (Ala^{267}–C-terminus) has been identified previously. Here, we applied additional tryptic digestion followed by oxidative cross-linking to identify the products originating from the N-terminal part of the α-subunit. These fragments start at Ala^{72} or Thr^{74} and contain the transmembrane H_1-H_2 domain. Formation of cross-linked product between α-fragments containing H_1-H_2 and H_7-H_{10} demonstrate that the structural integrity of the membrane moiety is preserved. We also determined that secondary cleavage of the 83-kDa fragment leads to the formation of C-terminal 48-kDa α-fragments with multiple N-termini at Ile^{582}, Ser^{583}, Met^{584} and Ile^{585}.

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Key words: Na^+,K^+-ATPase; α-Subunit; Chymotrypsin; Trypsin; Cu^{2+}-phenanthroline; Oxidative cross-linking

1. Introduction

Na,K-ATPase is the intrinsic enzyme of the plasma membrane responsible for coupled active transport of Na^+ and K^+ in animal cells. The enzyme consists of α (~112 kDa) and β (~55 kDa) subunits found in equimolar ratio. The catalytic α-subunit contains the ATP-hydrolyzing center on the large cytoplasmic protrusion, and cation occlusion sites within the membrane moiety that most probably contains 10 transmembrane segments [1]. Limited proteolysis of native Na,K-ATPase, along with other approaches, has been used extensively to study the structural organization of the enzyme within the plasma membrane [2–4]. Analysis of functional alterations induced by selective proteolytic splits of the α-subunit which are characteristic of different ligand-induced conformations: Na^+ form (E1) and K^+ form (E2), has provided valuable information on structure-function relationships [2,5–8]. Of particular interest are the studies of Na,K-ATPase subjected to controlled chymotryptic digestion in NaCl medium which abolishes ATPase activity and substantially alters other functional parameters [2,5,6,10]. For example, recently we showed that the chymotrypsin-digested preparation exhibits a twice higher capacity for phosphorylation by ATP than the native enzyme, thus indicating that half of the α-subunits of the native enzyme are dormant with respect to phosphoenzyme intermediate formation [10]. Interpretation of this and other observations with respect to structure-function correlations is complicated, however, due to the uncertainties about the structure of the chymotrypsin-digested Na,K-ATPase. The only product of this cleavage that has been well characterized is the 83-kDa fragment of the α-subunit from Ala^{267} to the COOH-terminus [6,9]. Neither the expected 30-kDa N-terminal fragment containing membrane segments H_1 and H_2 nor any other smaller products of its further digestion were detected previously [9,10]. Based on these observations we suggested that perhaps chymotrypsin splits the Na,K-ATPase α-subunit not only at the Leu^{266}-Ala^{267} bond, but also at a number of other peptide bonds within the 30-kDa N-terminal region, leading to the formation of numerous heterogeneous peptide fragments that are difficult to detect by standard methods [11]. On the other hand, the possibility could not be excluded that relatively short chymotryptic peptides containing membrane segments H_1 and H_2 were released from the postchymotryptic membrane moiety. Partial or complete release of some intramembrane peptides from the membrane moiety of proteolytically dissected Na,K-ATPase has been documented earlier [12–14].

To clarify these uncertainties, we aimed to elucidate the peptide composition of the chymotrypsin-digested Na,K-ATPase. According to the procedure developed in [11,15], the chymotrypsin-digested Na,K-ATPase was subjected to additional extensive tryptic digestion followed by oxidative cross-linking in the presence of Cu^{2+}-phenanthroline complex. Analysis of the cross-linked products has allowed us to determine the structures of the N-terminal part of the Na,K-ATPase α-subunit resulting from controlled chymotryptic digestion. We also determined the structures of the relatively stable products of the secondary digestion of the C-terminal 83-kDa peptide.

2. Materials and methods

The purified membrane-bound Na^+,K^+-ATPase of canine kidney medulla, with a specific activity of 1400–1600 μmol ATP hydrolyzed/mg protein/h, was prepared and assayed as described before [16]. Controlled chymotrypsin digestion was performed essentially as described by Jorgensen and Petersen [5]. The enzyme (0.5 mg/ml) was incubated at 37°C for 1 h in a solution containing 10 mM NaCl, 15 mM Tris-HCl (pH 7.4), and 25 μg/ml of α-chymotrypsin (Sigma, type II, bovine pancreas). The reaction was stopped by the addition of soybean trypsin-chymotrypsin inhibitor (Sigma): 100 μg/25 μg of chymotrypsin. The chymotrypsin-dissected Na,K-ATPase was collected by centrifugation at 160 000×g for 1 h at 4°C, and washed with 10 mM Tris-HCl (pH 7.4). Samples were subjected to electrophoresis on SDS polyacrylamide gels (7.5%) at pH 2.4 and stained with Coomassie [11]. For N-terminal sequence analysis peptide material from the 48-kDa band was transferred onto a polyvinylidene difluoride (PVDF) membrane.

Extensive trypsin digestion of native Na,K-ATPase or chymotryp-

*Corresponding author. Fax: (1) (419) 383-2871.
E-mail: NModyanov@Cutter.Mco.Edu

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PII S0014-5793(97)01493-2