Biochimica et Biophysica Acta, 1108 (1992) 247–252

BBAMEM 70707

Rapid Report

Occlusion of Rb^+ after extensive tryptic digestion of shark rectal gland Na,K-ATPase

Mikael Esmann [a] and Lars Sottrup-Jensen [b]

[a] *Institute of Biophysics, University of Aarhus, Aarhus (Denmark) and* [b] *Institute of Molecular Biology, University of Aarhus, Aarhus (Denmark)*

(Received 28 April 1992)

Key words: ATPase, Na^+/K^+-; Potassium ion occlusion; Rubidium ion occlusion; Occlusion; (Shark rectal gland)

Na,K-ATPase from rectal glands of *Squalus acanthias* has been subjected to proteolysis with trypsin. The E_1- and E_2-forms of the enzyme can be distinguished from the inactivation patterns at low trypsin concentrations, as previously seen with kidney enzyme. Extensive degradation by trypsin in the presence of 5 mM Rb^+ yields membrane fragments with a 19 kDa peptide as the major proteolytic fragment of the α-subunit. The sequence of the N-terminal 40 residues of this peptide is almost identical to that of a similar proteolytic fragment isolated by Capasso et al. (Capasso, J.M., Hoving, S., Tal, D.M., Goldshleger, R. and Karlish, S.J.D. (1992) J. Biol. Chem. 267, 1150–1158) using kidney Na,K-ATPase. Rb^+ occlusion can be fully retained under these circumstances, supporting the findings with kidney enzyme that only minor parts of the α-subunit are required to form a functional occlusion-site.

The purpose of the present experiments is to show that recent findings by Karlish and co-workers [1–3] on the structural components of the cation occlusion sites of Na,K-ATPase from pig kidney also pertain to Na,K-ATPase isolated from an entirely different species, *Squalus acanthias* (the spiny dogfish). The extensive trypsinization of Na,K-ATPase from pig kidney leaves a large fragment (about 19 kDa) in the membrane together with several minor components (less than 10 kDa). An important aspect is that occlusion-properties are retained in these fragments, which therefore must contain the cation binding sites of the enzyme [1–3]. The occlusion of cations is considered an important step in the mechanism of active transport of Na^+ and K^+ across the cell membrane. The result we present in this paper is that the structural features involved in Rb^+ occlusion are essentially the same for the shark enzyme as for the kidney enzyme. A 19 kDa fragment is the only major component in membrane fragments of shark enzyme with Rb^+ occlusion fully retained. The 40-residue N-terminal sequence is almost identical to that of the kidney enzyme (starting at threonine-834, Refs. 4 and 5) and a similar sequence of the *Torpedo californica* enzyme (starting at threonine-840, Ref. 6). A reason for our interest in the occlusion-properties of the shark enzyme is that detergent-solubilized Na,K-ATPase from shark is more resistant to inactivation by detergent than kidney enzyme [7]. Trypsinized shark enzyme could therefore form a good basis for experiments on isolation and purification of detergent-solubilized peptide fragments with retained Rb^+ occlusion ability.

Preparation of shark rectal gland enzyme. Na,K-ATPase from the rectal gland from *S. acanthias* was prepared as described by Skou and Esmann [8], but without the treatment with saponin. The Na,K-ATPase constituted about 70% of the protein (determined as the content of α- and β-subunits from SDS gel electrophoresis), and the specific activity was about 1700 μmol/mg protein per h. Na,K-ATPase and pNPPase activities, phosphorylation capacity (2.9 nmol/mg protein) and protein content were determined as previously described [9].

Ligand-dependent inactivation of Na,K-ATPase by trypsin. The inactivating effect of trypsin was followed in time according to the following protocol: 0.9 mg Na,K-ATPase protein/ml was incubated with RbCl or NaCl in 10 mM Tris/1 mM EDTA (pH 8.0) with trypsin (final concentrations 0.5 to 10 μg/ml). Aliquots

Correspondence to: M. Esmann, Institute of Biophysics, Ole Worms Allé 185, University of Aarhus, DK-8000 Aarhus, Denmark.

Abbreviations: E_1, the Na,K-ATPase conformation predominant in NaCl; E_2, the Na,K-ATPase conformation predominant in KCl (and RbCl); $C_{12}E_8$, octaethyleneglycol dodecyl monoether; pNPPase, K^+-dependent *p*-nitrophenylphosphatase; α-subunit, the 112 kDa catalytic subunit; β-subunit, the 35 kDa glycosylated subunit; PTH, phenylthiohydantoin; PVDF, poly(vinylidene difluoride).

were diluted 10-fold into an ice-cold buffer containing 100 μg trypsin inhibitor/ml, and residual Na,K-ATPase and pNPPase activities were assayed (at optimum conditions at 37°C, see Ref. 9).

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was done according to the method of Schagger and Von Jagow [10] using 1-mm 16.5% gels. Samples were freed from trypsin and trypsin inhibitor by centrifugation and delipidated before electrophoresis as described by Capasso et al. [3]. In brief the protein was dissolved in 2% SDS and 4 volumes of methanol was added at 0°C. The precipitated protein

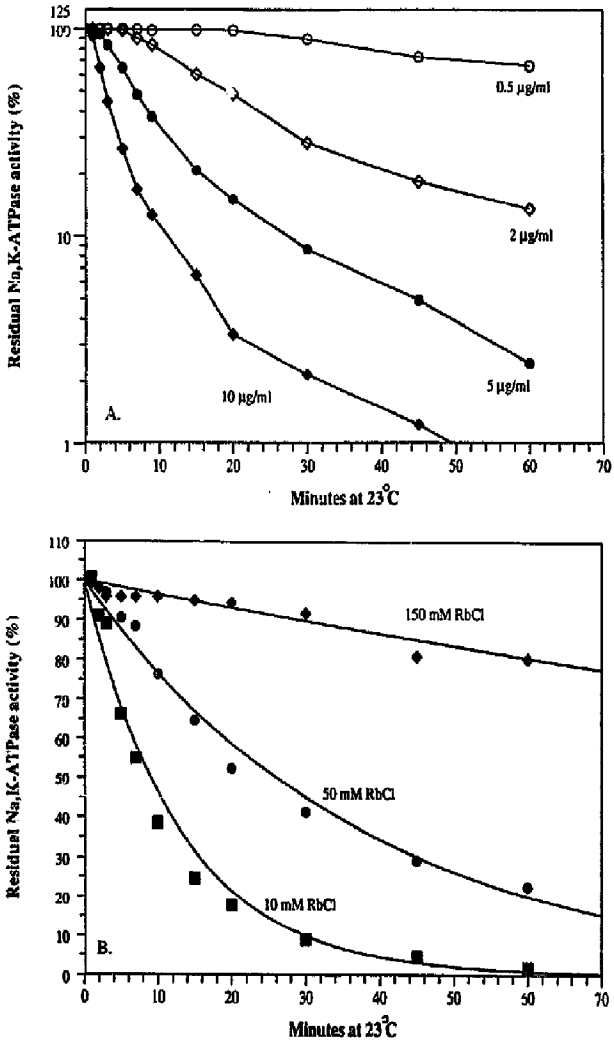

Fig. 1. Trypsin-dependent inactivation of shark Na,K-ATPase. In the experiments shown in panel A, 0.9 mg protein/ml was incubated in 10 mM Tris/1 mM EDTA (pH 8.0)/10 mM RbCl with trypsin. Final trypsin concentrations were 0.5 (open circles), 2 (open diamonds), 5 (filled circles or 10 μg/ml (filled diamonds). At the indicated time-points aliquots were diluted 10-fold into an ice-cold buffer containing 100 μg trypsin inhibitor/ml, and the residual Na,K-ATPase activities were assayed. In panel B the trypsin concentration was 5 μg/ml, and the RbC concentration was 10 (filled squares), 50 (filled circles) or 150 mM (filled diamonds). The full lines represent single-exponential fits of the form $Y(t) = 100 \cdot e^{-k \cdot t}$, with rate constants of 0.0036, 0.027 and 0.077 min^{-1}, respectively (top to bottom).

was centrifuged after 12 h at −20°C, and the pellet dissolved in sample buffer, which contained 100 mM sodium phosphate (pH 7.7), 1% 2-mercaptoethanol, 2% SDS and 36% urea. Samples were heated to 100°C for 5 min before electrophoresis. Staining was done in a solution of 0.25% Coomassie blue R250/40% ethanol/10% acetic acid for 1 h, and destaining done in 30% ethanol/10% acetic acid. Electroblotting was carried out as described by Matsudaira [11] using a LKB 2051 Midget Multiblot apparatus, and PVDF membranes were Problott (Applied Biosystems). Only HPLC-grade solvents and Millipore filtered or twice-destilled water were used. The electroblotting buffer [11] contained 0.005% SDS.

Sequenator analysis. Edman degradations were carried out on a 477A instrument from Applied Biosystems with on-line analysis of the phenylthiohydantoin (PTH) amino acids by reverse-phase HPLC on a 120A liquid chromatograph. The electroblotted samples were cut in small pieces and placed in the cross-flow chamber. The instrument was operated according to the instructions given by the manufacturer.

Measurement of Rb^+ occlusion using the cation-exchange procedure. The method used is essentially as described by Glynn and Richards [12]. The carboxylic resin Bio-Rad Bio-Rex 70 is equilibrated in 100 mM Tris (pH 7.0) and 1 mM EDTA. Na,K-ATPase (0.9 mg/ml) in 30 mM histidine (pH 7.0) and 1 mM CDTA is pre-incubated with 5 mM $^{86}Rb^+$ at 23 or 37°C for 5 min. Trypsin is added to give final concentrations of 0.125 or 0.5 mg/ml, and at the desired time-point an aliquot is rapidly cooled to 0°C. About 450 μl is then immediately forced through a 1-ml ion exchange column at 2°C, with the speed of the piston being adjusted to allow the enzyme suspension to be in contact with the resin for 2.5 s (this is calculated from the flow-rate and the volume of the liquid-phase in the resin). The amount of ^{86}Rb emerging from the column is determined from γ-radiation. The specific radioactivity is adjusted to give about 350 cpm per nmol Rb^+.

Materials. Trypsin (T-8642), trypsin inhibitor (T-9128) and molecular weight markers (MW-SDS-17S and MW-SDS-70) were obtained from Sigma.

Results and Discussion. Na,K-ATPase from shark is susceptible to inactivation by trypsin in low concentrations, Fig. 1 (Panel A). The kinetics of inactivation at 23°C are not following a single-exponential decay curve, in contrast to the behaviour of the kidney Na,K-ATPase [13], but the major part of the activity is lost rapidly and in proportion to the trypsin concentration. With 50 μg trypsin/ml or more (not shown) the Na,K-ATPase activity is completely abolished within the first minute. The susceptibility to inactivation depends very much on the ionic strength, with a high ionic strength (e.g., 150 mM RbCl, Fig. 1B) offering considerable protection towards trypsin. The previously observed ligand-dependent pattern of inactivation [13] can also be seen with the shark enzyme, Fig. 2. Here is shown the

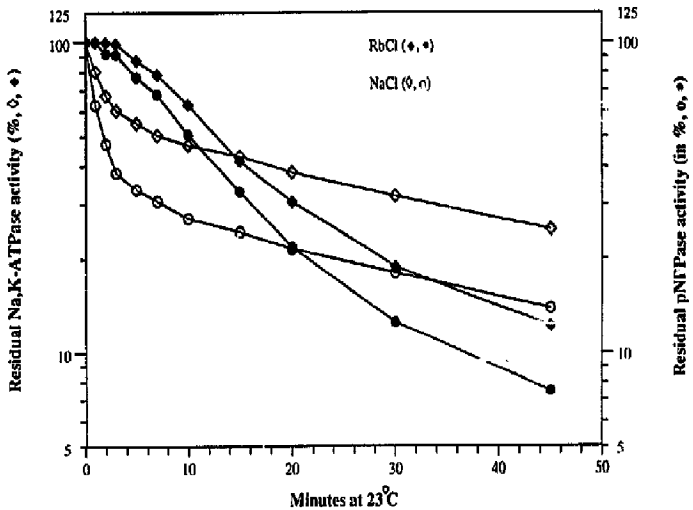

Fig. 2. Ligand-dependent inactivation of shark Na,K-ATPase by trypsin. 0.9 mg protein/ml was incubated in 10 mM Tris/1 mM EDTA (pH 8.0) with 3.5 μg trypsin/ml and with 10 mM RbCl (filled symbols) or 10 mM NaCl (open symbols). At the indicated time-points aliquots were diluted 10-fold into an ice-cold buffer containing 100 μg trypsin inhibitor/ml, and the residual Na,K-ATPase (diamonds) and pNPPase activities (circles) were assayed.

inactivation pattern of both the Na,K-ATPase activity and the K^+-dependent pNPPase activity when proteolysis is carried out with enzyme in the E_1-form (10 mM NaCl) or in the E_2-form (10 mM RbCl). In NaCl, the inactivation is markedly biphasic with time, with the pNPPase activity being lost more rapidly than the Na,K-ATPase activity. In RbCl, there is an almost parallel loss of the two activities, and the time-course is clearly different from that in NaCl. With RbCl there is a small lag period followed by a rapid loss of activity. The proteolytic digestion proceeds essentially via the same routes as described for kidney enzyme [13], with two fragments having molecular masses about 50 and 40 kDa being produced by the tryptic split in the presence of RbCl. With NaCl a small amount of 80 kDa-peptide is formed (data not shown). The digestion by trypsin is done at low ionic strength (10 mM RbCl or NaCl) because the sensitivity to trypsin is more marked at low ionic strength (Fig. 1B) and also because the differences between the ligand-dependent time-courses of loss of activity is more clear at low ionic strength (not shown). We have also chosen to perform the inactivation at 23°C rather than 37°C to avoid instability of the shark enzyme at higher temperatures, which is more pronounced at low ionic strength.

Fig. 3 shows the stability of Rb^+ occlusion after extensive tryptic digestion. When digestion is carried out at 23°C, there is an increase in the occlusion capacity with time (up to 8.2 nmol/mg protein at 60 min), an increase which is more rapid the higher the trypsin concentration. Performing the digestion at 37°C leads to loss of occlusion capacity with time, Fig. 3. The data shown in Fig. 3 gives the total amount of Rb^+ carried through the cation-exchanger, i.e., both the specifically occluded Rb^+ and the non-specific binding. In the presence of 2.5 mM ADP, where specific occlusion is abolished in the native enzyme, the non-specific binding at 5 mM Rb^+ is about 1.5 nmol/mg protein. There is thus a large, specific occlusion capacity retained even after total loss of overall enzymatic activity (which is complete within a minute at these trypsin concentrations). The increase in occlusion capacity could be due to a higher affinity for Rb^+ of the trypsinized membranes, as seen also by Karlish et al. [1], or due to an increase in non-specific binding. However, the fact that the occlusion capacity decreases when digestion is performed at 37°C suggests that the increase in occlusion capacity is due to an increased affinity for Rb^+ rather than an increased non-specific Rb^+-binding. The stoichiometry between the number of Rb^+ occlusion sites and phosphorylation sites is about 2.3 Rb^+ per phosphorylation site, which indicates that there is full saturation of the two Rb^+-sites at the concentration of 5 mM RbCl used in the present experiments. Solubilization in $C_{12}E_8$ of trypsinized membranes decreases the amount of specifically oc-

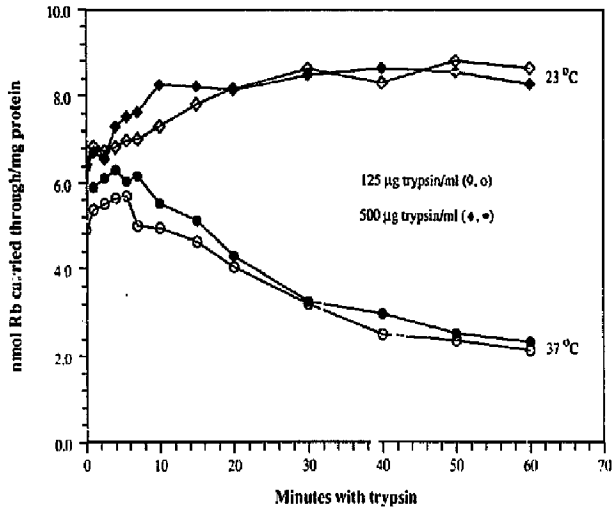

Fig. 3. Stability of occlusion of Rb^+ during trypsinolysis of shark Na,K-ATPase. Enzyme (0.9 mg/ml) was preincubated with 5 mM $^{86}Rb^+$ for 5 min and the ability to occlude Rb^+ was followed with time after addition of trypsin (125 µg/ml, open symbols, or 500 µg/ml, filled symbols) either at 23°C (diamonds) or 37°C (circles). At the desired time-points an aliquot was cooled to 0°C and passed though an ion exchange column (see text). From the radioactivity emerging from the column the amount of Rb^+ occluded was calculated (in nmol per mg protein applied to the column).

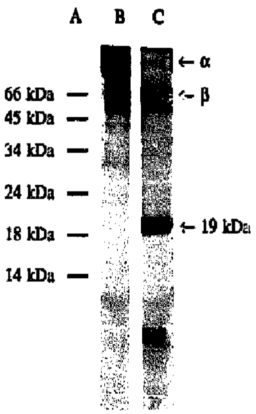

Fig. 4. Digestion of shark rectal gland Na,K-ATPase by trypsin. Enzyme was digested with 0.5 mg trypsin/ml for 60 min at 23°C, and prepared for electrophoresis as described in the text. Lane A, positions of molecular weight markers (66, 45, 34, 24, 18.4 and 14.3 kDa, respectively); Lane B, control enzyme (no trypsin added, loaded with approx. 50 μg protein); Lane C, trypsinized Na,K-ATPase. The positions of the α- and β-subunits are marked, as is that of the 19 kDa tryptic fragment.

cluded Rb^+ to about 2.1 nmol/mg, i.e., about 30% of the value for the membrane-bound trypsinized enzyme (not shown).

Fig. 4 shows a gel-pattern of trypsinized membranes, compared with a control enzyme (note that the lane with control enzyme has been heavily overloaded with protein to detect minor components in addition to the α- and β-subunits). The dominant band in the trypsinized membranes (marked 19 kDa) is absent in the control enzyme, and the β-subunit is virtually unaffected by trypsin, as seen also with the kidney enzyme [2]. Several minor bands are present in the 10 kDa range in the trypsinized membranes, these have not been described further. In agreement with the observations by Karlish et al. [1], the tryptic fragment of 19 kDa is not found to an appreciable extent if the proteolysis is carried out in the absence of Rb^+.

The N-terminal amino acid sequence of the 19 kDa band was determined for a 40-residue stretch, which in Table I is compared with published sequences of the α-subunit from *Torpedo californica*, pig kidney and sheep kidney (the complete amino acid sequence of the shark enzyme not known yet). The sequences in this 40-residue stretch are almost identical. The position of the tryptic split in the shark enzyme suggests that it occurs corresponding to positions 840 in the *Torpedo* enzyme [6] and position 834 in the kidney enzymes [4,5]. It is interesting that the tryptic split in pig kidney enzyme occurs at position 831 in the kidney enzyme, and that a combination of trypsin and pronase forms a tryptic fragment of 18.5 kDa starting at residue 834 [3].

In conclusion, the present experiments suggest that occlusion of Rb^+ by Na,K-ATPase from shark rectal gland is governed by parts of the α-subunits which are highly homologous in structure to those of the kidney enzyme. It is also shown that the E_1- and E_2-forms of the enzyme can be distinguished in the shark enzyme using the trypsin technique. Further experiments employing detergent solubilization of the occlusion sites may give more detailed information on the protein structures necessary for cation occlusion.

The authors wish to thank Ms. Birthe Bjerring Jensen and Ms. Angielina Tepper for excellent technical assistance, and Jesper V. Møller for advice on the gel electrophoresis experiments and Steven J.D. Karlish for helpful suggestions. Financial support was received from the Danish Medical Research Council

TABLE I

N-terminal sequence of the 19 kDa tryptic fragment of shark rectal gland Na,K-ATPase

The sequence is compared with the published amino acid sequences from *Torpedo californica* [6], pig kidney [4] and sheep kidney [5]. Amino acid identities are marked with an asterisk. The putative tryptic split is marked with a:, and the first residue after the split corresponds to amino acid numbers 840 (*Torpedo*) or 834 (pig and sheep kidney, in all three cases four residues before the site of the split is shown).

```
                  840
Torpedo   RNPK  : TDKLVNERLISMAYGQIGMIQALGGFFSYFVILAENGFLP

                   ********** ****************************
                  1   5    10        20        30        40
Shark^a   XXXX  : MDKLVNERLISIAYGQIGMIQALGGFFSYFVILAENGFLP

                   ********** *************** ************
                  834
Pig       RNPK  : TDKLVNERLISMAYGQIGMIQALGGFFTYFVILAENGFLP

           ***    ******************************** *******
                  834
Sheep     RNPQ  : TDKLVNERLISMAYGQIGMIQALGGFFTYFVIMAENGFLP
```

[a] In step 28 minor amounts of PTH-Gly were also detected. In step 35 PTH-Asp and PTH-Glu were present in equimolar yields. The yield of PTH-Met in step 1 was 167 pmol.

(Grant No. 12-8245 to M.E.), Aarhus University Research Foundation and from the Danish Biomembrane Research Center.

References

1 Karlish, S.J.D., Goldshleger, R. and Stein, W.D. (1980) Proc. Natl. Acad. Sci. USA 87, 4566–4570.

2 Karlish, S.J.D., Goldshleger, R., Tal, D.M. and Stein, W.D. (1991) in The Sodium Pump: Structure, Mechanism and Regulation (De Weer, P. and Kaplan, J.H., eds.). Vol. 46 (Part I), pp. 129–141, Rockefeller University Press, New York.

3 Capasso, J.M., Hoving, S., Tal, D.M., Goldshleger, R. and Karlish, S.J.D. (1992) J. Biol. Chem. 267, 1150–1158.

4 Ovchinnikov, Yu. A., Arsenyan, S.G., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Aldanova, N.A., Arzamazova, N.M., Arystarkhova, E.A., Melkov, A.M., Smirnov, Yu. V., Guryev, S.O., Monastyrskaja, G.S. and Modyanov, N.N. (1985) Proc. Acad. Sci. USSR 235, 1490–1495.

5 Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.

6 Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1985) Nature 326, 733–736.

7 Esmann, M. (1986) Biochim. Biophys. Acta 857, 38–47.

8 Skou, J.C. and Esmann, M. (1979) Biochim. Biophys. Acta 567, 436–444.

9 Esmann, M. (1988) Methods Enzymol. 156, 105–115.

10 Schagger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 369–379.

11 Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.

12 Glynn, I.M. and Richards, D.E. (1982) J. Physiol. 330, 17–43.

13 Jorgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415.