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Identification of the segment of the catalytic subunit of (Na^+,K^+)ATPase containing the digitalis binding site

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Digitalis compounds that are extensively used in the treatment of cardiovascular disorders are known to bind specifically at the extracellular side of (Na^+,K^+)ATPase. We have recently reported the synthesis of [^{3}H]*p*-nitrophenyltriazene-ouabain, a derivative of ouabain, which specifically alkylates the catalytic chain of the (Na^+,K^+)ATPase at a defined region of the sequence. The peptidic segment involved in the binding of digitalis to (Na^+,K^+)ATPase has been located after mild trypsin treatment of the labeled enzyme. In the presence of 100 mM KCl, tryptic fragmentation results in two peptide fragments of mol. wt. 58 000 and 41 000, respectively. The radioactive probe labeled only the 41 000 fragment indicating that the digitalis binding site is located on the 41 000 domain situated at the N-terminal part of the sequence of the α-subunit.

Key words: affinity labeling/digitalis site/peptide mapping

Introduction

Digitalis compounds have great value in the treatment of chronic congestive heart failure and other disorders associated with the cardiovascular system. In addition to its clinical importance, this family of molecules has attracted the interest of biochemists, physiologists, and biophysicists because of its action on the trans-membranal transport of sodium and potassium. It is well known that the sodium pump in the plasma membrane of eukaryotic animal cells is inhibited by low concentrations of cardiac glycosides (Schatzman, 1953).

The (Na^+,K^+)ATPase is composed of two subunits, a large polypeptide (α) of mol. wt. ~94 000 and a small glycoprotein (β) of mol. wt. ~57 000. The functional unit of the (Na^+,K^+)ATPase is probably arranged in an $\alpha_2\beta_2$ complex (Kyte, 1972; Jørgensen, 1974a; Perrone *et al.*, 1975). The catalytic subunit of the (Na^+,K^+)ATPase spans the phospholipid bilayer exposing the cardiac glycoside site at the extracellular surface of the plasma membrane and the active site for ATP hydrolysis at the cytoplasmic side.

Various conditions have been discovered under which different specific patterns of cleavage of the α-subunit occur during limited proteolysis (Castro and Farley, 1979; Jørgensen, 1977). Analysis of the different tryptic and chymotryptic fragments obtained in this way from the labeled enzyme has already permitted the mapping of both the phosphorylation site (on an intra-cytoplasmic sequence) by the use of γ-[^{32}P]ATP, and the hydrophobic intra-membrane sequence by the use of [^{3}H]adamantane diazirine (Farley *et al.*, 1980) or 5-[^{125}I]iodonaphthylazide (Karlish *et al.*, 1977).

Our aim was to localize the extracellular segment of the peptide bearing the digitalis site. The topological localization of this peptidic domain could be useful in the elucidation of the three-dimensional structure of the α-subunit, once its complete sequence is known. Covalent labeling of the cardiac glycoside receptor site was achieved with [^{3}H]*p*-nitrophenyltriazene-ouabain (NPT-ouabain), a covalent derivative of ouabain which reacts specifically with the α-subunit of the (Na^+,K^+)ATPase (Rossi *et al.*, 1980).

Results

Trypsin cleavage of the α-subunit of (Na^+,K^+)ATPase from rabbit kidney

Different conformational states of the (Na^+,K^+)ATPase purified from rabbit and dog kidney yield different specific hydrolysis products after partial tryptic digestion (Castro and Farley, 1979; Jørgensen, 1977). In the presence of KCl, the catalytic subunit (mol. wt. 94 000) is cleaved into smaller peptides which appear on SDS gels as two doublets near mol. wts. 58 000 and 41 000. The 41 000 mol. wt. fragment is subsequently digested to a 37 000 fragment. In the presence of NaCl, or in the absence of any alkali cations, a mild tryptic digestion generates a 77 000 mol. wt. fragment. The cleavage patterns we obtained with the rabbit kidney enzyme are presented in Figure 1B and C. They confirm previous results obtained by other authors except that in the absence of both NaCl and KCl minor bands of mol. wt. 38-45 000 and 58 000 appeared under our conditions. Interestingly, the ouabain-bound enzyme gave the same tryptic pattern in the presence of KCl as the free enzyme (Figure 1D).

Castro and Farley (1979) reported that the ATP phosphorylation site of the (Na^+,K^+)ATPase from canine kidney medulla is located on a peptide overlapping the 77 000 and 41 000 fragments, as indicated in Figure 2. The experiments to locate the phosphorylated peptides obtained by trypsinolysis of the rabbit kidney enzyme, are presented in Figure 3. They show that the radioactivity was incorporated in both the 41 000 and 77 000 fragments. Thus, the location of the phosphorylated segment in the α-subunit is identical for the canine kidney and rabbit kidney enzymes. The peptide map for the rabbit kidney enzyme is the same as that proposed by Castro and Farley (1979) for the dog kidney enzyme (Figure 2).

Covalent labeling of (Na^+,K^+)ATPase with [^{3}H]NPT-ouabain

The digitalis site of rabbit kidney (Na^+,K^+)ATPase was covalently labeled with NPT-ouabain. Maximal covalent labeling of the (Na^+,K^+)ATPase was reached after an incubation of 2 h with 5 μM [^{3}H]NPT-ouabain, and it was equivalent to 12% of the total specific ouabain binding capacity, as measured with [^{3}H]ouabain.

The radioactive profile presented in Figure 4A shows that the (Na^+,K^+)ATPase from rabbit kidney was selectively labeled on the catalytic subunit (mol. wt. 94 000) as was found previously for (Na^+,K^+)ATPase obtained from a variety of other sources (Rossi *et al.*, 1980). After mild tryptic digestion in the presence of KCl, the radioactive probe was found on the 41 000 mol. wt. fragment, and no radioactivity was incorporated in the 58 000 mol. wt. doublet. It was not possible to detect any specific covalent incorporation of

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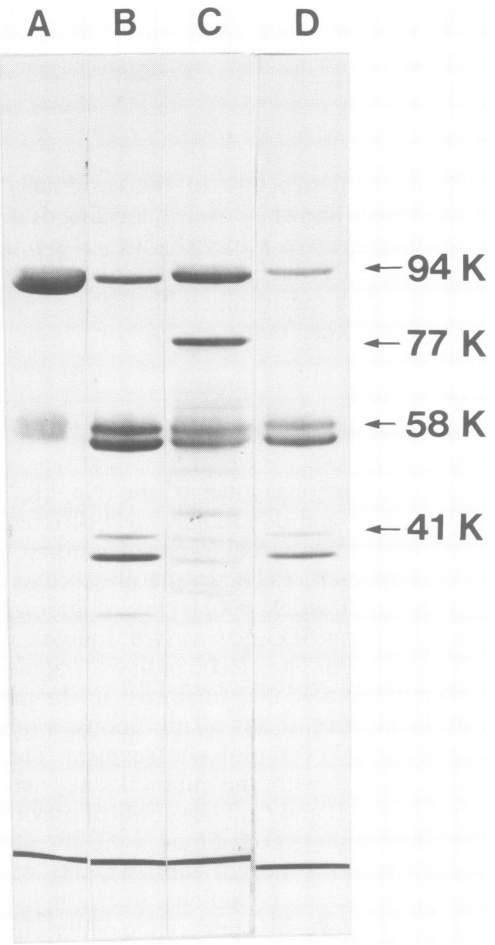

Fig. 1. Peptides separated by polyacrylamide gel electrophoresis and stained with Coomassie blue. **A.** Intact purified rabbit kidney (Na^+,K^+)ATPase. **B.** Enzyme digested in the presence of KCl. The digestion was performed for 5 min at 25°C with 2 mg/ml ATPase and 0.2 mg/ml DPCC-treated trypsin in 25 mM imidazole/HCl, 100 mM KCl, and 1 mM EDTA (pH 7.5). **C.** Enzyme digested in the absence of KCl. The (Na^+,K^+)ATPase (2 mg/ml) was incubated for 2 min at 37°C with 150 μg/ml of DPCC-treated trypsin. **D.** Ouabain-bound enzyme digested in presence of KCl. (Na^+,K^+)ATPase was previously incubated in the presence of 1 mM ouabain and 5 mM $MgCl_2$ before digestion as indicated in **B**. The digestions were stopped by the addition of a 2-fold excess of soybean trypsin inhibitor. The samples were then applied to a Laemmli (1970) electrophoretic system. The same patterns of tryptic digestion were obtained when (Na^+,K^+)ATPase previously labeled with [^{3}H]NPT-ouabain, was used.

[^{3}H]NPT-ouabain into (Na^+,K^+)ATPase that had been previously treated with trypsin in the absence of NaCl and KCl. This result indicates that after tryptic digestion under these conditions the α-subunit must be in a conformation that renders the ouabain binding site inaccessible to [^{3}H]NPT-ouabain, although it can still be phosphorylated by γ-[^{32}P]ATP.

Discussion

The digitalis binding site was shown to be in the α-subunit sequence of the (Na^+,K^+)ATPase from the distribution of radioactivity found in the different fragments presented in Figures 4A and 4B. In the presence of KCl, no labeling was observed in the 58 000 mol. wt. doublet that represents the C-terminal part of the α-chain: radioactive labeling occurred only in the N-terminal 41 000 fragment (Figure 4B). Interestingly, the 41 000 fragment contains two sites that are

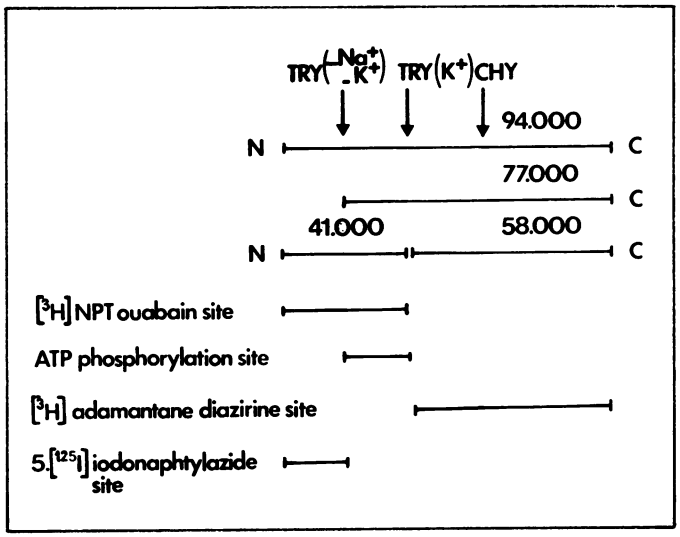

Fig. 2. Summary of the mapping of various sites within the α-subunit sequence. The location of the sites reacting with γ-[^{32}P]ATP (Castro and Farley, 1979), [^{3}H]adamantylidene (Farley *et al.*, 1980) and 5-[^{125}I]iodonaphthylazide (Karlish *et al.*, 1977) have been reported previously. The location of the [^{3}H]NPT-ouabain site was determined from the data described in the text. TRY and CHY refer to a trypsin and chymotrypsin cleavage, respectively. The ionic conditions of tryptic digestion are shown in brackets.

important for the function of the enzyme: (i) the site that is phosphorylated by ATP and that is known to be exposed on the cytoplasmic side of the membrane; and (ii) the site specific for digitalis binding that is known to be exposed at the external face of the membrane.

It is shown in Figure 2 that the ouabain binding site is located on the 41 000 N-terminal fragment. The location of peptides labeled by hydrophobic probes such as [^{3}H]adamantane diazirine (Farley *et al.*, 1980) and 5-[^{125}I]iodonaphthylazide (Karlish *et al.*, 1977) are presented for comparison in Figure 2.

Materials and methods

Materials

Ouabain and diphenyl carbamyl chloride (DPCC)-treated trypsin were obtained from Sigma. Electrophoresis-grade acrylamide, methylenebisacrylamide, SDS and N,N,N′,N′-tetramethylethylenediamine as well as soybean trypsin inhibitor were supplied by Serva. [^{3}H]Ouabain and Aquassure were purchased from New England Nuclear and γ-[^{32}P]ATP was an Amersham product.

Preparation of (Na^+,K^+)ATPase

Purified (Na^+,K^+)ATPase was prepared from the red outer medulla of rabbit kidneys using the SDS treatment described by Jørgensen (1974b). The specific (Na^+,K^+)ATPase activity was 20–25 μmol/min/mg of protein at 37°C and the maximal capacity of [^{3}H]ouabain binding sites was 2500 pmol/mg of protein. The purified enzyme was stored at −70°C in a 25 mM imidazole-HCl buffer (pH 7.4) containing 1 mM EDTA.

Labeling of (Na^+,K^+)ATPase with [^{3}H]NPT-ouabain

[^{3}H]NPT-ouabain was synthesized as previously described (Rossi *et al.*, 1980). (Na^+,K^+)ATPase (0.3 mg/ml) was incubated with 5 μM of [^{3}H]NPT-ouabain (0.6 Ci/mmol) at 25°C in 1 ml of a solution containing 50 mM triethanolamine-HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, and 2 mM ATP. After 2 h, the samples were centrifuged at 220 000 *g* for 45 min, and the pellets washed once with 25 mM imidazole-HCl (pH 7.4) containing 1 mM EDTA before being used for gel electrophoresis or digestion with trysin. To estimate the non-specific labeling, a parallel experiment was performed in the presence of a large excess (1 mM) of unlabeled ouabain, as previously described (Rossi *et al.*, 1980).

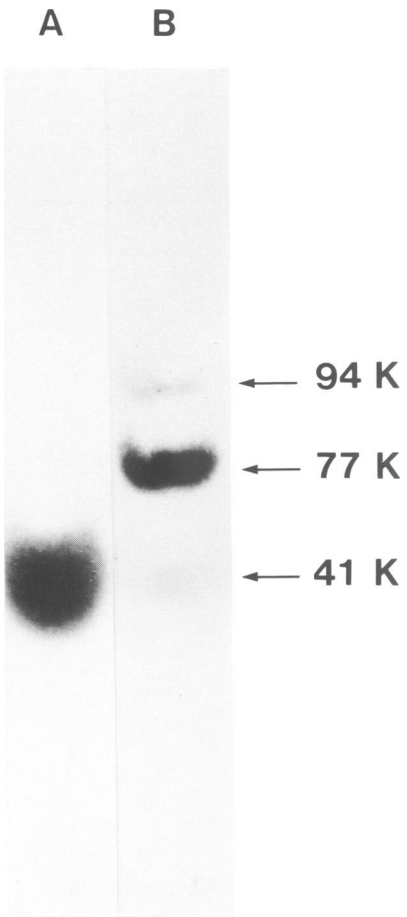

Fig. 3. Identification of the phosphorylated fragments of (Na^+,K^+)-ATPase. ATPase was phosphorylated with γ-[^{32}P]ATP in 5 mM $MgCl_2$, 100 mM NaCl, 25 mM imidazole, and 1 mM dithiothreitol (pH 7.4), for 30 s at 25°C. Samples were then precipitated in 5% TCA before being applied to polyacrylamide gels prepared according to the Avruch and Fairbanks (1972) electrophoresis system. **A.** Autoradiograms of phosphorylated fragments of (Na^+,K^+)ATPase after a mild digestion in the presence of KCl. **B.** Autoradiograms of fragments obtained from enzyme pre-digested in the absence of both NaCl and KCl prior to incubation with γ-[^{32}P]ATP. Conditions of tryptic digestion of **A** and **B** are described in the legend to Figures 1B and 1C, respectively.

Phosphorylation with γ-[^{32}P]ATP

Purified (Na^+,K^+)ATPase (70 μg) was incubated at 25°C for 30 s with 2 mM γ-[^{32}P]ATP (20 Ci/mmol) in 50 μl of a solution containing 25 mM imidazole-HCl (pH 7.4) 2 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol. The phosphorylation was performed either before or after proteolytic digestion. The reaction was started by addition of γ-[^{32}P]ATP and was stopped by the addition of 10 volumes of ice-cold trichloracetic acid (TCA) (5%). After centrifugation at 3000 *g* for 10 min, the pellet was washed once with 1 ml TCA (5%) and twice with water before solubilization.

Proteolysis

Purified (Na^+,K^+)ATPase was cleaved with DPCC-treated trypsin in 25 mM imidazole-HCl (pH 7.4) containing 1 mM EDTA. In the absence of alkali cations, the trypsin/ATPase ratio used was 1:130, while in the presence of 100 mM KCl this ratio was 1:10. The reaction was stopped by addition of a 2-fold molar excess of soybean trypsin inhibitor relative to trypsin.

Gel electrophoresis

^{3}H-labeled fragments were separated on analytical 10% SDS polyacrylamide gels according to Laemmli (1970). Mol. wts. were determined using bovine serum albumin (mol. wt. 68 000), catalase (58 000), ovalbumin (45 000), lactate dehydrogenase (33 000), chymotrypsinogen (25 000), and myoglobin (17 800). After staining using the procedure of Fairbanks *et al.* (1971), the gels were sliced into 3-mm thick sections, which were incubated at 80°C in 0.3 ml of 30% H_2O_2 for 16 h. Five ml of Aquassure (New England Nuclear) was then added to each vial and the extracted radioactivity was measured in a Packard Tri-Carb 3390 spectrometer. A higher recovery of tritium from gel slices was obtained when the gels were run without β-mercaptoethanol. The ^{32}P-phosphorylated fragments were separated on a SDS polyacrylamide slab gel using the buffer system of Avruch and Fairbanks (1972) at pH 2.4. Slabs were then dried and the phosphorylated fragments were localized by autoradiography using Kodak X-O Mat S film.

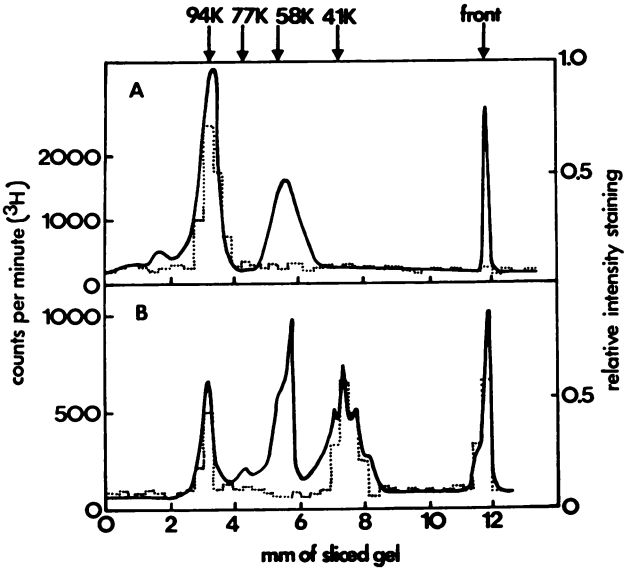

Fig. 4. Gel scans and distribution of radioactivity in fragments of intact and digested (Na^+,K^+)ATPase after labeling with [^{3}H]NPT-ouabain. **A.** Intact purified enzyme. **B.** Radioactive profile after a graded tryptic digestion of the labeled enzyme in the presence of KCl. The (Na^+,K^+)ATPase was incubated for 2 h at 25°C with 5 μM [^{3}H]NPT-ouabain. Samples were then centrifuged and washed in 25 mM imidazole, 1 mM EDTA (pH 7.4) before being digested as described in the legend of Figure 1. The solid line represents the relative intensity of staining and the dotted line the specific radioactive labeling. Specific radioactivity was calculated for each band by subtracting the radioactivity of a sample incubated with 1 mM of unlabeled ouabain before addition of [^{3}H]NPT-ouabain from the radioactivity of a sample incubated in the presence of [^{3}H]NPT-ouabain.

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