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Structural Studies on H^+, K^+-ATPase: Determination of the NH_2-Terminal Amino Acid Sequence and Immunological Cross-Reactivity with Na^+,K^+-ATPase

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The NH_2-terminal amino acid sequence of the 100 kilodalton subunit of porcine gastric H^+,K^+-ATPase has been determined to be YKAENYELYQVELGPGP. Although the NH_2-terminal region of this protein is not similar to the same region of the lamb kidney Na^+,K^+-ATPase catalytic subunit, other regions of these ATPase proteins appear to be homologous. Both monoclonal and polyclonal antibodies raised to lamb kidney Na^+,K^+-ATPase and its α, but not β, subunit cross-react with the 100 kilodalton protein of H^+,K^+-ATPase.

The plasma membrane Na^+,K^+-ATPase, the gastric H^+,K^+-ATPase and the sarcoplasmic reticulum Ca^{++}-ATPase are thought to be closely related because they each contain a catalytic subunit of approximately 100 kDa, have a common 4 amino acid sequence at the site of acyl phosphorylation (1), and an identical 5 amino acid sequence at the fluorescein isothiocyanate labeling site (2). Recently the complete amino acid sequences, derived from cDNA nucleotide sequences, were reported for the catalytic subunits of Na^+,K^+-ATPase (3,4) and sarcoplasmic reticulum Ca^{++}-ATPase (5,6). However, relatively little is known about the structure of the H^+,K^+-ATPase. The determination of the NH_2-terminal sequence of the gastric H^+,K^+-ATPase is of particular interest since Shull et al. (3) have suggested that the hydrophilic NH_2-terminal region of the Na^+,K^+-ATPase and Ca^{++}-ATPase may be involved in the cation selectivity of these enzymes. Furthermore, there is some question as to

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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whether the gastric enzyme is an oligomer of identical or nonidentical 100 kDa proteins (ref. 7 for review), and whether it has any immunologic similarity to the plasma membrane Na^+,K^+-ATPase (8).

In the present study we purified the catalytic subunit of the gastric H^+,K^+-ATPase, determined its NH_2-terminal amino acid sequence, and demonstrated substantial immunological cross-reactivity between the catalytic subunits of the two enzymes using polyclonal and monoclonal antibodies raised against the lamb kidney Na^+,K^+-ATPase.

EXPERIMENTAL PROCEDURES

Enzyme Preparations. Na^+,K^+-ATPase was purified from frozen lamb kidneys as previously described (9). H^+,K^+-ATPase was prepared from fresh porcine gastric mucosal scrapings essentially as described by Chang et al. (10), except that the tissue was homogenized in a Waring blender and then with a Polytron PT35; and the discontinuous gradients of 7% Ficoll and 37% sucrose in 25 mM imidazole, pH 7.5, were run in a Beckman 45 Ti fixed angle rotor at either 150,000 x g (avg) for 4 hrs or at 125,000 x g for 17 hrs. With this homogenization procedure very little protein or enzyme activity was recovered at the sample-7% Ficoll interface. The material at the Ficoll-37% sucrose interface (G_{II}) was suspended in 0.25 M sucrose in 25 mM imidazole, pH 7.5, pelleted by centrifugation at 150,000 x g, resuspended in the same buffer and stored at -20°C.

Assays. K^+-dependent p-nitrophenyl phosphatase activity was determined at 37°C by continuous measurement at 410 nm. The reaction medium contained 25 mM histidine, pH 7.4, 5 mM $MgCl_2$, 4 mM p-nitrophenyl phosphate, with and without 10 and 20 mM KCl and 0.15 mM ouabain. K^+-dependent ATPase was measured at 37°C in a medium containing 25 mM histidine, pH 7.4, 5 mM $MgCl_2$, 5 mM Na_2ATP (Boehringer Mannheim), with and without 10 mM KCl and 0.15 mM ouabain. Inorganic phosphate was measured by a modification of the method of Fiske and SubbaRow (11). ATPase activity was also measured using the spectrophotometric, linked enzyme Na^+,K^+-ATPase assay system described previously (12). Protein was measured by the method of Lowry et al. (13).

Subunit purification and sequencing. The 100 kDa protein of the H^+,K^+-ATPase was purified by gel filtration on a 1.5 cm x 190 cm column of Bio Gel A-5m, equilibrated in 25 mM Tris-Cl, pH 7.5, 0.1% SDS and 0.02% NaN_3. Prior to chromatography, the gastric enzyme was solubilized by heating at 60°C for 20 min in 25 mM Tris-Cl, pH 7.5, 5% SDS and 1% 2-mercaptoethanol. The column eluant was monitored at 280 nm, and only those fractions which exhibited a single protein band of approximately 100 kDa upon SDS-PAGE (14) were pooled. The pooled fractions were dialyzed at 3°C for 48 hrs against 10 mM ammonium bicarbonate and lyophilized prior to sequencing. Another technique was also used to purify H^+,K^+-ATPase catalytic protein for sequencing. The enzyme was first labeled with fluorescein isothiocyanate exactly as described previously for the Na^+,K^+-ATPase (15), solubilized with 10 mM sodium phosphate, pH 7.4, 3% SDS and 0.1% 2-mercaptoethanol, and electrophoresed on a 7.5% SDS-polyacrylamide slab gel according to Weber and Osborn (16). The fluorescent 100 kDa band was excised from the gel and electroblotted onto GF/C paper that had been activated with trifluoroacetic acid (17) and then coated with 10 mg/ml Polybrene

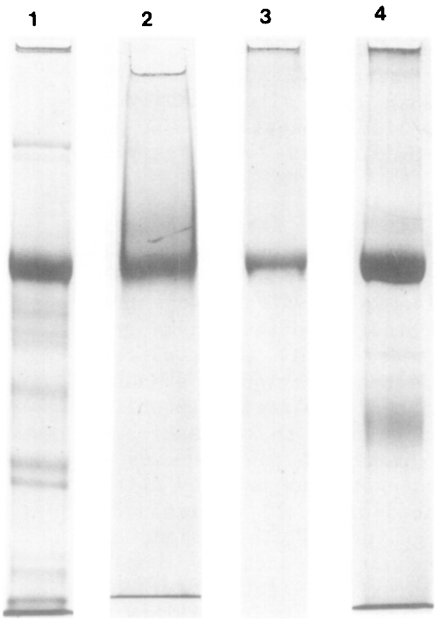
(Pierce), air dried, and the excess removed immediately before use by washing in H_2O (18). Electroblotting was carried out at 200 mA for 2hr in 25 mM Tris-Cl, pH 8.3, plus 0.5 mM dithiothreitol at 8°C. The fluorescent strip of GF/C paper was excised, washed with 12% MeOH, blotted dry and stored at -20°C until being inserted directly into the sequencer and sequenced with no precycling. Both H^+,K^+-ATPase catalytic protein preparations were sequenced on an Applied Biosystems 470A gas phase sequencer, and the phenylthiohydantoin-amino acids were analyzed on a Waters Nova-Pak C_{18} HPLC column.

Antibody production and measurement of binding. Rabbit antisera were raised against the native lamb kidney Na^+,K^+-ATPase and against its isolated and denatured and β subunits as previously described (19). Monoclonal antibodies raised against the native Na^+,K^+-ATPase were prepared as described previously (20). Antibody binding to the enzyme preparations was measured using three methods: 1) an indirect solid-surface absorption binding assay that has been previously standardized (19,20), in which the antigens were adsorbed to plastic microtiter plate wells (Cooke flexible plates), and then exposed to the anti-sera or monoclonal antibodies. A β-galactosidase-Protein A conjugate or β-galactosidase sheep anti-mouse IgG $(F_{ab'})_2$ conjugate was used to detect the antigen-bound rabbit or mouse IgG, respectively. 2) an immunodot assay in which the antigens were adsorbed to nitrocellulose paper and the paper then exposed to antibody. The bound antigen-antibody complex was then detected using either horse radish peroxidase-goat anti-rabbit IgG or a peroxidase-goat anti-mouse IgG conjugate. 3) Western blots of electrophoretically resolved proteins transferred to nitrocellulose sheets (Schleicher and Schuell) and then exposed to the various antisera as described by Towbin et al. (21).

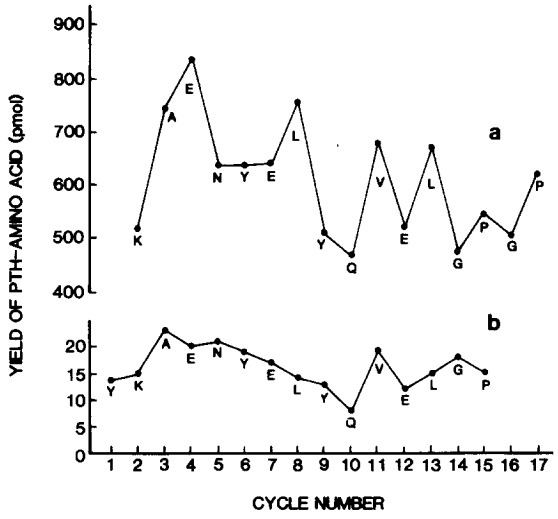
RESULTS AND DISCUSSION

The H^+,K^+-ATPase G_{II} membrane fraction that was used in this study exhibited K^+-dependent, ouabain-insensitive p-nitrophenyl phosphatase and ATPase activities of 16.7 μmol/mg/hr and 65 μmol/mg/hr, respectively. Neither activity was significantly altered by prior incubation of the enzyme with 0.015% SDS, indicating that the enzyme-containing vesicles are not impermeable to the reaction ligands. The addition of 0.15 mM ouabain to the phosphatase and ATPase assay media revealed the presence of ouabain-sensitive, K^+-dependent activities corresponding to 2.6% (ATPase) and 11.5% (phosphatase) of the total. When the gastric fraction was assayed under conditions optimal for the Na^+,K^+-ATPase, 7% of the total ATPase activity was inhibited by 0.15 mM ouabain.

The protein profile of the enzyme preparation on SDS-PAGE is shown in Fig. 1. Although the enzyme is not pure, a fraction containing essentially a single 100 kDa protein band was prepared by SDS-gel filtration of the G_{II} fraction on Bio Gel A-5m. After dialysis, the 100



(1)



(2)

Fig. 1 Gel electrophoresis of enzyme and subunit fractions. SDS-polyacrylamide gels (7.5% acrylamide, 0.75 mm thick) were prepared according to Laemmli (14) run in a Hoefer Mighty Small Unit, and stained with Coomassie Blue R. lane 1, 10 µg H^+,K^+-ATPase G_{II}; lane 2, 3 µg column purified 100 kDa protein from G_{II}; lane 3, 2.5 µg Na^+,K^+-ATPase α subunit; lane 4 10 µg lamb kidney Na^+,K^+-ATPase.

Fig. 2 Sequence analysis of H^+,K^+-ATPase. The yield of phenylthiohydantoin (PTH)-amino acids at each cycle of Edman degradation is shown for the 100 kDa protein of H^+,K^+-ATPase. Panel a, column-purified 100 kDa protein shown in lane 2 of Fig. 1; Panel b, 100 kDa protein electroblotted onto derivatized GF/C paper. The initial coupling yield for the column-purified sample was 33%.

kDa protein fraction was subjected to NH_2-terminal amino acid sequencing (Fig. 2a). Due to high background levels in the initial cycles, the NH_2-terminal residue could not be positively identified. However, subsequent sequencing of the 100 kDa protein which had been electroblotted onto GF/C paper and then washed with 12% MeOH (Fig. 2b), unambiguously identified the amino acid in cycle 1 as tyr and verified the sequence shown in Fig. 2a out to cycle number 15. It is apparent from these results that the NH_2-terminus of the gastric H^+,K^+-ATPase is not blocked, as had been reported previously by Peters et al. (22), who used

the less sensitive dansyl chloride procedure. There was no evidence of a second sequence in this fraction. Therefore, if the H^+,K^+-ATPase is an oligomer of nonidentical 100 kDa proteins, they have the same NH_2-terminal sequence. In addition, the NH_2-terminal sequence of the H^+,K^+-ATPase is not homologous with either the Na^+,K^+- or Ca^{++}-ATPases, which supports the suggestion by Shull et al (3) that the NH_2-terminal regions of these ATPase proteins are involved in cation selectivity.

To compare other regions of the H^+,K^+-ATPase and Na^+,K^+-ATPase proteins, the ability of antibodies raised against the lamb kidney Na^+,K^+-ATPase and its purified subunits to cross-react with the H^+,K^+-ATPase was investigated. Rabbit polyclonal and four monoclonal antibodies directed against the native Na^+,K^+-ATPase showed essentially no cross-reactivity with native H^+,K^+-ATPase using either the solid-surface adsorption assay or the immunodot procedure. However, Western blot analysis (Fig. 3) showed that both Na^+,K^+-ATPase- and α subunit-directed rabbit antisera bind to the 100 kDa protein of the H^+,K^+-ATPase, while β subunit-directed antibodies show little if any binding to the H^+,K^+-ATPase preparation. In addition, two of the four monoclonal antibodies (M7-PB-E9, and M8-P1-A3) also bind to the electroblotted H^+,K^+-ATPase. These results indicate that, although there is no apparent immunological similarity between the native forms of these two membrane-bound ATPases, there are substantial homologies between the denatured catalytic proteins, and that the H^+,K^+-ATPase does not contain a β-like subunit. Therefore, although the H^+, K^+-ATPase and Na^+,K^+-ATPase enzymes have different tertiary structures or altered conformations, the amino acid sequences at many determinant sites of the 100 kDa proteins must be similar.

Our results are in sharp contrast to the report by Peters et al. (8) that goat antisera prepared against the rabbit kidney Na^+,K^+-ATPase and its isolated α and β subunits did not cross-react with the porcine H^+,K^+-ATPase. They also reported that antisera raised to SDS-denatured

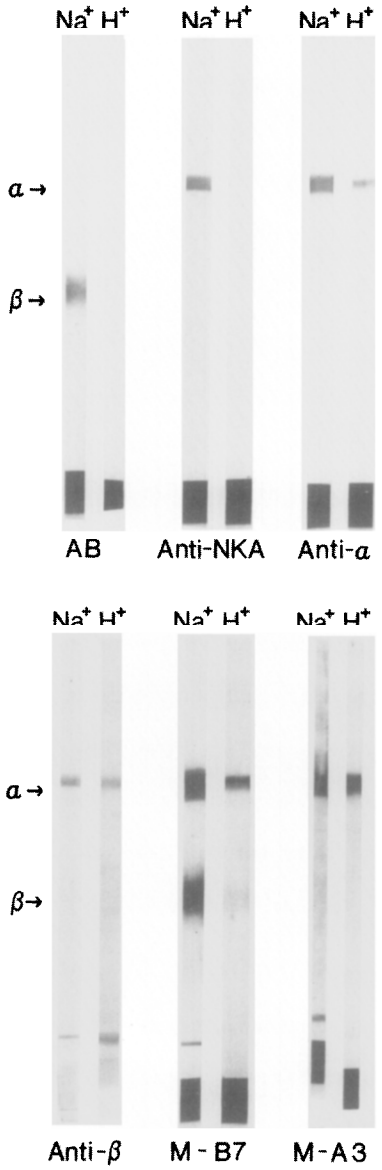


Fig 3. Western blot analysis of the specificity of various Na^+,K^+-ATPase-directed antibodies. Na^+,K^+-ATPase (100 µg) and H^+,K^+-ATPase, G_{II} (200 µg) were subjected to SDS-PAGE on 14 cm slab gels, transferred to nitrocellulose and incubated with antiserum or monoclonal antibodies. Bound antibody was detected with peroxidase-conjugated second antibody. The first two strips (upper left) show amido black staining of the blotted Na^+,K^+-ATPase (Na^+) and H^+,K^+-ATPase (H^+). Subsequent pairs of strips (Na^+,H^+) demonstrate binding of rabbit Na^+,K^+-ATPase-directed antibodies (Anti-NKA); α-directed antibodies (Antiα), β-directed antibodies (Anti-β), and monoclonal antibodies MIO-P6-B7 (M-B7) and M8-P1-A3 (M-A3), to the Na^+,K^+-ATPase and H^+,K^+-ATPase, respectively. Representative blots of two different anti-α, two anti-β, the two nonreactive (M10-P6-B7, M12-P7-F11) and two reactive (M8-P1-A3, M7-PB-E9) monoclonal antibodies are shown.

H^+,K^+-ATPase had no effect on Na^+,K^+-ATPase activity and concluded that there was no cross-reactivity. The latter observation is not unexpected in view of our present results. Their inability to detect immunological cross-reactivity between the two enzymes by Western blot analysis could be explained by their use of ^{125}[I]-labeled protein A to identify antigen-bound goat antibodies, since staphylococcal protein A is known to show rather wide variations in its ability to bind to goat IgG (23).

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