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Sequence analysis of the catalytic subunit of H^+-ATPase from porcine renal brush-border membranes

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The catalytic subunit of the H^+-ATPase from brush-border membranes of porcine renal proximal tubules was labeled with the hydrophobic SH-group reagent 10-*N*-(bromoacetyl)amino-1-decyl-β-glucopyranoside (BADG) which irreversibly inhibits proton pump avitivity in the absence but not in the presence of ATP. The labeled protein was purified and digested with proteinases. After isolation and sequencing of proteolytic peptides two BADG-labeled cysteines were identified. The amino acid sequences of the obtained proteolytic peptides were homologous to the catalytic subunit of V-ATPases. From mRNA of porcine kidney cortex a catalytic H^+-ATPase subunit was cloned. 181 of the 183 amino acids which overlap in the sequence derived from the cDNA and the proteolytic peptides were identical, and the two deviations are due to single base exchanges. A comparison of the amino acid sequence derived from the cloned cDNA with sequences of catalytic H^+-ATPase subunits communicated by other laboratories revealed 98%, 96% and 94% identity with sequences from bovine adrenal medulla, from bovine kidney medulla and from clathrin-coated vesicles of bovine brain. Between 64% and 69% identity was obtained with sequences from fungi and plants. The data show that the catalytic subunit of V-ATPases is highly conserved during evolution. They indicate organ and species specificity in mammalians.

Introduction

ATP-driven proton pumps have been identified in renal cortex and medulla [1–7]. They belong to the vacuolar types of ATPases (V-ATPases) which can be distinguished from other types of ATPases by their insensitivity to vanadate and oligomycin and by their sensitivity to *N*-ethylmaleimide and nitrate [6,8–15]. Employing functional studies and immunohistochemistry in kidney V-ATPases have been localized in luminal membranes of proximal and distal tubules, in luminal and basal membranes of intercalated cells of collecting ducts and in membranes of intracellular vesicles [1,3,6]. Since it is assumed that V-ATPases contain three essential subunits with molecular masses around 70 kDa, 60 kDa and 17 kDa [16–18] and the purification of V-ATPases from bovine kidney revealed additional polypeptides with apparent molecular weights of 45000, 42000, 38000, 33000, 31000, 14000 and 12000 [2,9], different subtypes of V-ATPase may exist in different parts of the nephron and/or in different subcellular localizations. These subtypes may differ with respect to the structure of the essential three subunits and/or may contain different additional subunits [19,20].

The catalytic subunit with a molecular weight of about 70000 [16,21] which is homologous in V-ATPases from phylogenetically different species [22–24] should be a component of all V-ATPase subtypes in mammals and its primary structure should be highly conserved in different species, organs and/or subcellular localizations. Whereas species and organ specificity of the catalytic subunit can be investigated by cDNA cloning, sequence differences of the catalytic subunits in different subcellular localizations can only be determined by amino acid sequencing of subunits which are purified from different cellular compartments.

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Abbreviations: BADG, 10-*N*-(bromoacetyl)amino-1-decyl-β-glucopyranoside; TFA, trifluoroacetic acid; PCR, polymerase chain reaction.

In the present study the primary structure of the catalytic subunit of a V-ATPase was investigated which is localized in brush-border membranes of proximal tubules from pig kidney. Therefore, brush-border membrane vesicles were isolated from renal cortex, the catalytic subunit was affinity labeled and then isolated and partially sequenced. With the exception of two amino acid residues the obtained amino acid sequences were identical to the amino acid sequence derived from the catalytic H^+-ATPase subunit which was cloned from mRNA of porcine kidney cortex.

Materials and Methods

Materials

2'-Desoxyadenosine 5-[α-[^{35}S]thio]triphosphate (50 TBq/mmol) was delivered by Du Pont de Nemours (Dreieich, Germany) and *Taq* polymerase by Perkin Elmer Cetus (Langen, Germany). *Escherichia coli* XL1-Blue cells and pBluescriptII SK plasmid were supplied by Stratagene (Heidelberg, Germany). V8 proteinase (2.2 units/mg), diisopropylfluorophoshate, FITC-labeled anti-rabbit IgG antibodies from goat and the molecular mass marker proteins myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and myoglobin fragments (10.6 kDa, 8.2 kDa, 6.2 kDa) were obtained from Sigma (München, Germany). Gold-labeled anti-rabbit IgG antiserum from goat was purchased from Amersham Buchler (Braunschweig, Germany), poly(vinylidene difluoride) (Immobilon) membranes from Millipore (Eschborn, Germany) and Acridine orange from Eastman Kodak (Rochester, NY, USA). Trypsin, sequencing grade, and restriction enzymes were delivered by Boehringer (Mannheim, Germany). pUC18 plasmid, Superscript MMLV reverse transcriptase, Klenow polymerase and T_4 polynucleotide kinase were supplied by Bethesda Research Laboratories (Eggenstein, Germany), and the T7-sequencing kit by Pharmacia (Freiburg, Germany). All other chemicals were obtained as described earlier [25].

Preparation of brush-border membrane vesicles

Brush-border membrane vesicles from porcine renal cortex were prepared by a Ca^{2+}-precipitation method, resuspended and frozen in liquid nitrogen [26]. The right-side out oriented vesicles [27] were transformed to inside-out oriented vesicles by incubation with deoxycholate as recently described by Simon and Burckhardt [5].

Precipitation of membrane-associated proteins

Brush-border membrane vesicles were solubilized with 0.4% (w/v) deoxycholate in the presence of 1 M D-glucose and membrane-associated proteins were precipitated by reducing the detergent concentration as described earlier [26]. The precipitate (PI) was isolated by 1 h centrifugation at 200000 × *g*. These steps were performed in the presence of the proteinase inhibitors phenylmethylsulfonyl fluoride (1 mM), diisopropylfluorophosphate (0.25 mM), leupeptin (10 μM), aprotinin (2 μM) and benzamidine (1 mM).

Measurements of proton uptake

ATP-dependent proton uptake into inside-out oriented vesicles was measured with the pH-sensitive dye Acridine orange as described by Sabolić and co-workers [28]. Briefly 30 μl of vesicles were added to a cuvette containing 1 ml (37°C) of 50 mM Hepes-Tris (pH 7.0), 150 mM KCl, 5 mM $MgCl_2$ plus 6 μM Acridine orange. After excitation at 492 nm, the emission at 540 nm was measured until a stable base line was obtained. Then, 10 μl of 100 mM ATP-Tris (pH 7.0) was added and the change of emission at 540 nm was measured.

Labeling of brush-border membrane proteins with BADG

Precipitated brush-border membrane proteins (PI) and inside-out oriented brush-border membrane vesicles were covalently labeled with ^{14}C-labeled 10-*N*-(bromoacetyl)amino-1-decyl-β-glucopyranoside (BADG) as described before [29]. Briefly PI was washed three times with 20 mM imidazole cyclamate (pH 8.5), 0.1 mM magnesium cyclamate, 100 mM potassium cyclamate (KC buffer) and suspended at a protein concentration of 5 mg per ml in 20 mM imidazole cyclamate (pH 8.5), 0.1 mM magnesium cyclamate, 100 mM sodium cyclamate (NaC buffer). Then 50 μM [^{14}C]BADG was added and the suspension was incubated for 1 h at 37°C. The reaction was stopped by addition of 100 mM cysteine, PI sedimented by 20 min centrifugation at 250000 × *g* and resuspended in 0.1 M Tris-HCl (pH 7.4). Inside-out vesicles (2 mg protein per ml) were labeled by incubation for 30 min (37°C) in NaCl buffer containing 50 μM [^{14}C]BADG plus either 5 mM $MgCl_2$ or 5 mM MgATP. Then free [^{14}C]BADG and ATP was removed on a small Sephadex G-50 column.

Solubilization and fractionation of PI

For solubilization [^{14}C]BADG-labeled PI (30 mg of protein) was incubated 1 h (37°C) in 10 ml of 0.1 M Tris-HCl (pH 7.4) containing 5% (w/v) SDS and the above described proteinase inhibitors. The suspension was centrifuged 20 min (22°C) at 200000 × *g* and the clear supernatant was applied to a 1.8 m long Bio-Gel A column with an internal diameter of 2.7 cm. The column was equilibrated with 0.1 M Tris-HCl (pH 7.4) plus 1% (w/v) SDS and run at a flow rate of 15 ml per h. Fractions containing protein plus radioactivity were dialyzed against water (48 h, room temperature), con-

centrated by lyophilization and suspended in sample buffer of SDS-polyacrylamide gel electrophoresis.

Purification of the catalytic H^+-ATPase subunit

The final purification of the M_r 70000 polypeptide was performed by separating fractions from the Bio-Gel A column in which the M_r 70000 polypeptide was clearly separated from other polypeptides on SDS-polyacrylamide gels. Concentrated fractions from the Bio-Gel A column containing 0.5 to 1 mg of protein were applied on surfaces of 1.5 mm thick SDS-polyacrylamide slab gels (14 cm × 20 cm) which were run as described by Laemmli [30]. After electrophoresis part of the gel was stained with Coomassie brilliant blue R250 and the M_r 70000 polypeptide was dissected and electroeluted from the unstained gel as described before [29]. The eluted protein was dialyzed for 24 h (22°C) against H_2O containing 0.01% (w/v) SDS and frozen.

Immunological procedures

To prepare polyclonal antibodies, two rabbits were immunized on day 1 and 28 by subcutaneous injection of 30 μg of the electroeluted M_r 70000 polypeptide with complete and incomplete Freund's adjuvant, respectively. Immune reactions on Western blots were performed as described earlier [31] and for immunohistochemistry previously described procedures [32,33] were modified. For light microscopy rat kidneys were fixed in Carnoy's fluid (ethanol/chloroform/acetic acid, 6:3:1, v/v), embedded in paraffin, cut into 5-μm sections and dewaxed. The sections were blocked by incubating 15 min (22°C) with PBS containing 1% (w/v) bovine serum albumin, 0.2% (w/v) Tween 20 and 0.1% (w/v) Triton X-100 and incubated with rabbit antiserum which was diluted (1:20) in the same buffer. To visualize the antibody binding, the sections were incubated 45 min with FITC-labeled anti-rabbit IgG antibodies from goat (1:30) in PBS containing 1% (w/v) BSA. For electron microscopy the reaction with the primary antibody (antiserum diluted 1:30) on ultrathin, LR White resin-embedded tissue sections was performed exactly as described before [33]. The immune reaction of the primary antibodies was visualized with gold-labeled anti-rabbit IgG antiserum from goat (1:30). No immune reaction was observed when the antisera were replaced by preimmune sera.

Proteolytic digestion and fractionation of proteolytic peptides

For digestion with V8 proteinase from *Staphylococcus aureus*, 50 μg of the electroeluted [^{14}C]BADG-labeled M_r 70000 polypeptide were incubated 16 h (37°C) in 300 μl of 100 mM ammonium acetate (pH 4.0) containing 0.6% (w/v) SDS plus 300 μg of V8 proteinase. The digested sample was vacuum-concentrated to 50 μl, the pH was adjusted to 6.8 and 50 μl of electrophoresis sample buffer [30] was added. The peptides were separated by SDS electrophoresis on 16% (w/v) polyacrylamide gels which was performed as described by Schägger and Von Jagow [34]. After electrophoresis the peptides were electrically transferred to poly(vinylidene difluoride) membranes by employing semi-dry blotting [35]. Then the membranes were washed with H_2O, wetted with destaining solution ($CH_3OH/H_2O/CH_3COOH$, 6:3:1, v/v), stained (2 s) with staining solution ($CH_3OH/H_2O/CH_3COOH$, 4.5:4.5:1, v/v, plus 0.02% (w/v) Amido black) and finally washed with destaining solution. The stained peptides were dissected and used for amino acid sequencing.

Trypsin digestion of the [^{14}C]BADG-labeled M_r 70000 polypeptide was performed within dissected gel slices and the proteolytic fragments were eluted [36]. After SDS-polyacrylamide gel electrophoresis the M_r 70000 polypeptide was identified (5 min staining with Coomassie brilliant blue R250 and destaining for 2 h in $H_2O/CH_3OH/CH_3COOH$, 6:3:1, v/v) and carefully dissected. The gel pieces were extensively washed with H_2O, frozen, partially freeze-dried and then 5 h (37°C) incubated in 2 ml of 100 mM NH_4HCO_3 (pH 8.5) containing 0.5 mM $CaCl_2$ and 40 μg trypsin. The proteolytic fragments were extracted by incubating the gel pieces three times; first with 75% (v/v) trifluoroacetic acid (TFA) and then with a 1:1 (v/v) mixture of TFA and acetonitrile (each incubation was for 4 h at room temperature). The extracted solutions were combined, vacuum-concentrated and applied to reverse-phase HPLC on a C_{18} column (Lichrospher 100 RP-18, Merck, Darmstadt, Germany) which was equilibrated with H_2O containing 0.1% (v/v) TFA (gassed with helium). The sample was eluted with a gradient between H_2O plus 0.1% (v/v) TFA and 80% (v/v) acetonitrile, 19.9% H_2O and 0.1% (v/v) TFA (gassed with helium). The eluted polypeptides were detected at 206 nm.

Amino acid sequencing

Sequencing was carried out on a gas phase sequencer (470A, Applied Biosystems) which was equipped with an on-line analyser for phenylthiohydantoin derivatized amino acids using a standard program supplied by Applied Biosystems.

cDNA cloning and sequencing

Oligonucleotides were synthesized in 0.2 μmol scale on a Cyclon Plus DNA synthesizer from MilliGen (Eschborn, Germany) using the solid phase phosphoamidite method and further purified by HPLC.

For the polymerase chain reaction (PCR) poly(A)-enriched RNA from pig kidney cortex was prepared [37]. Oligo(dT)-primed single stranded cDNA was syn-

thesized and two internal H^+-ATPase-specific cDNA fragments were amplified by PCR employing primer pairs which were derived from amino acid sequences of proteolytic peptides. For the first fragment a degenerated primer pair (see $S1^+$ and $S2^-$ in Table I) was deduced from peptide sequences 2 and 9 (see Table II, YGIVNEN and AEMPAD, respectively). The PCR reaction was performed in a 100 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 mM of $S1^+$ and 4 mM of $S2^-$. After an initial denaturation step for 5 min at 94°C, 2.5 units *Taq* polymerase were added and 35 amplification cycles (1 min 94°C, 2 min 50°C, 3 min 72°C) were performed. The resulting amplification product was digested with *Xba*I and subcloned in pUC18 [38]. For amplification of the second cDNA fragment one degenerated oligonucleotide (see $S4^-$ in Table I) deduced from peptide sequence 13 (see Table II, AFYDM) was used in combination with an oligonucleotide (see $S3^+$ in Table I) which was deduced from the previously determined cDNA sequence. The reaction was initiated and *Taq* polymerase was added as described above and 35 cycles each consisting of 45 s at 94°C, 1 min at 52°C and 2 min at 72°C were performed. The amplification product was digested with *Eco*RI and again subcloned in pUC18.

To amplify the 5'-end of the catalytic H^+-ATPase subunit cDNA was synthesized from mRNA employing $S5^-$ (Table I) as internal primer. The amplification was performed employing the oligonucleotides $S6^-$ and $S7^+$ (Table I). $S6^-$ was derived from the previously determined sequence and $S7^+$ from the recently communicated cDNA sequence of the catalytic H^+-ATPase subunit of bovine adrenal medulla [39]. The PCR-reaction was performed and the amplified PCR-fragment was digested with *Eco*RI and subcloned in pUC18 as described above.

To determine the 3'-end of a catalytic H^+-ATPase subunit from porcine kidney cortex the 3'-RACE method [40] was employed. After priming the mRNA with the oligo(dT) containing adapter primer $S8^-$ (Table I) cDNA was synthesized and a cDNA-fragment was amplified with the adapter oligonucleotide $S9^-$ and the oligonucleotide $S10^+$ which was derived from the cDNA determined earlier (Table I). In these experiments the PCR reaction was performed in the presence of 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.17 mg/ml of bovine serum albu-

TABLE I

Nucleotide sequences of oligonucleotides which were used for PCR experiments

The nucleotide positions within the coding region of the cDNA (according to Fig. 7) are shown above each oligonucleotide. + and − indicates whether the sequences belong to the plus or minus DNA strand. Positions in which the degenerated code was considered are indicated by strokes (/). Sequences which encode the additional *Xba*I or *Eco*RI restriction sites are underlined.

	493 512	
S1+	5'GG TCT AGA TAC/T GGA/C/G/T ATA/C/T GTA/C/G/T AAC/T GAA/G AA3'	
	1148 1132	
S2-	5'GG TCT AGA TC A/C/G/TGC A/C/G/TGG CAT C/TTC A/C/G/TGC3'	
	1093 1108	
S3+	5'CCG GAA TTC AGA TGG GCT GAG GCC C3'	
	1685 1672	
S4-	5'CC GGA ATT CAT A/GTC A/GTA A/GAA A/C/G/TGC3'	
	699 682	
S5-	5'CAG CTT CTC AGT GAC AGG3'	
	533 513	
S6-	5'C CGG AAT TCT GTG TTT GAT GAG CGA G3'	
	1 17	
S7+	5'CCG GAA TTC TGC ACC TCG CGC CC3'	
S8-	5'GACTCGAGTCGATCGAT$_{(17)}$3'	
S9-	5'GACTCGAGTCGATCG3'	
	1530 1547	
S10+	5'G GCT TCC TA GCA GAA AC3'	
	1564 1581	
S11+	5'GAG GTA GCA AAG CTT ATC3'	
	1593 1609	
S12+	5'C CTA CAG CAA AAT GGA T3'	

min, 5% (v/v) dimethyl sulfoxide, 1.5 mM each dNTP, and 0.6 μM each primer. After initial denaturation and addition of *Taq* polymerase 35 PCR cycles (1 min 94°C, 90 s 50°C, 3 min 72°C) were performed and the reaction mixture was diluted 100-fold with H_2O. Then a second PCR reaction of 35 cycles was performed in which the adapter oligonucleotide S9$^-$ and a second H^+-ATPase related oligonucleotide (S11$^+$, Table I) were used. The resulting amplification product was hybridized with oligonucleotide S12$^+$ (Table I) and gel-eluted. After polishing the termini with Klenow polymerase and after phosphorylation with T_4 polynucleotide kinase the amplification product was subcloned blunt end into the pBluescriptII SK plasmid [38].

The DNA sequences of the different subcloned cDNA fragments were determined by the dideoxy chain termination method [41] using the T7 DNA polymerase and 2'-deoxyadenosine 5-[α-[^{35}S]thio]triphosphate. Both strands of the cDNA inserts were sequenced using universal primers of pUC18 and pBluescriptII SK. The cDNA sequences were verified by sequencing two recombinant plasmids originating from two independent PCR reactions.

Results

Labeling of the catalytic H$^+$-ATPase subunit from the brush-border of proximal tubules

Previously components of porcine renal Na^+/D-glucose cotransporter with molecular weights around 75000 were labeled with the covalently binding D-glucose analog BADG [29]. The polypeptides labeled with BADG revealed a broad band in SDS-polyacrylamide gels. Since 30% of the BADG labeled peak was protected by the addition of D-glucose, it was discussed whether or not BADG may also react with nonrelated polypeptides with molecular weights near 75000. One candidate was the catalytic subunit of the V-ATPase in brush-border membranes, since V-ATPases are inhibited by the SH-group reagent *N*-ethylmaleimide which binds to the catalytic subunit [12,16,42], and BADG is also an SH-group reagent. To investigate an interaction of BADG with the V-ATPase, we tested whether or not the ATP-dependent proton uptake over the brush-border membrane was inhibitable by BADG. The measurements were performed with purified brush-border membrane vesicles which were inverted by deoxycholate treatment so that proton uptake into the vesicles could be measured after addition of ATP from the outside [5]. Firstly, the vesicles were incubated 30 min (37°C) at pH 8.4 with 5 mM MgATP (control), 5 mM Mg^{2+} plus 50 μM BADG, or 5 mM MgATP plus 50 μM BADG. Then free BADG was removed and ATP-driven proton uptake was analysed by measuring the formation of pH gradients by Acridine orange fluorescence quenching. Fig. 1 shows that, after incubation with 50 μM BADG, ATP-driven proton uptake was inhibited by more than 98% and that this inhibition was completely prevented if 5 mM ATP was present. These data suggested that BADG binds to the catalytic subunit of the H^+-ATPase from brush-border membranes.

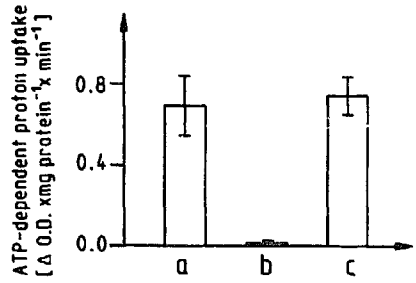

Fig. 1. Interaction of BADG with ATP-driven proton transport in brush-border membranes of proximal tubules. Everted brush-border membrane vesicles of porcine renal proximal tubules were incubated 30 min (37°C) in alkaline NaC buffer containing 5 mM $MgCl_2$ (a), 5 mM $MgCl_2$ plus 50 μM BADG (b) or 5 mM MgATP plus 50 μM BADG (c). Then free BADG and ATP was removed and ATP-dependent proton uptake into the vesicles was measured.

Purification of the catalytic subunit of the H$^+$-ATPase

To purify the BADG-labeled catalytic subunit for partial sequencing, a fraction of brush-border membrane proteins (PI) was prepared from porcine brush-border membrane vesicles in which membrane-associated proteins were enriched [26,43]. PI was labeled with 50 μM [^{14}C]BADG at pH 8.4 in the presence of 70 mM Na^+ as described before [29]. After the reaction was stopped with cysteine and free [^{14}C]BADG was removed, the labeled proteins were solubilized with SDS and separated by gel chromatography on a Bio-Gel A-1.5 column in the presence of 1% (w/v) SDS (see Materials and Methods). Fig. 2 shows the distribution of labeled and unlabeled polypeptides before and after fractionation by gel chromatography. Partial separation of polypeptides with apparent molecular weights between 65000 and 95000 was obtained. In this molecular weight range two [^{14}C]BADG-labeled polypeptides can be distinguished: one was strongly labeled with BADG, formed a sharp band and was eluted in fractions 36 to 41. The other showed less [^{14}C]BADG labeling, formed a broad band and was eluted in fractions 38–44. Since D-glucose protection experiments suggested that the broad band is the component of the Na^+/D-glucose cotransporter (data not shown) the sharp band with an apparent molecular weight of 70000 was considered as candidate for the catalytic subunit of the V-ATPase.

To isolate the BADG-labeled M_r 70000 polypeptide fractions 36 and 37 from Fig. 2 were collected, separated in preparative SDS-polyacrylamide slab gels and the M_r 70000 polypeptide was dissected. Un-

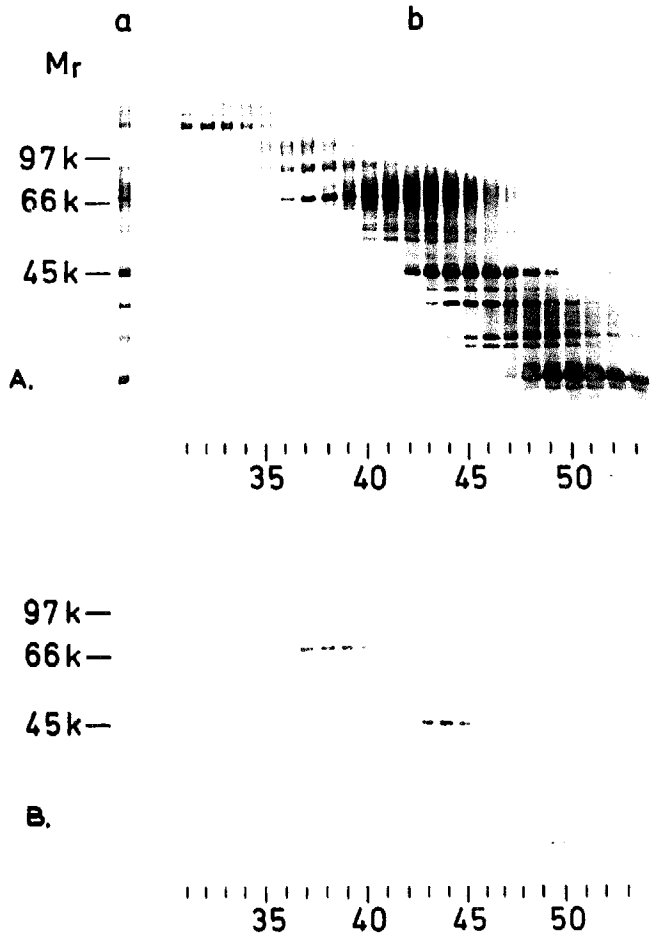

Fig. 2. Fractionation of BADG-labeled brush-border membrane proteins by gel chromatography. Purified porcine renal brush-border membrane vesicles were solubilized with deoxycholate and a protein fraction (PI) was isolated as described in Materials and Methods. PI was covalently labeled with [^{14}C]BADG and solubilized with 5% (w/v) SDS. The supernatant obtained after 20 min centrifugation at 200000 × g was fractionated on a Bio-Gel A column with 0.1 M Tris-HCl (pH 7.4) plus 1% (w/v) SDS as running buffer. The nonfractionated supernatant (a) and different fractions from the Bio-Gel A column (b) were applied to SDS-polyacrylamide slab gels. After electrophoresis the gels were either stained with silver (A) or dried and analyzed by autoradiography (B).

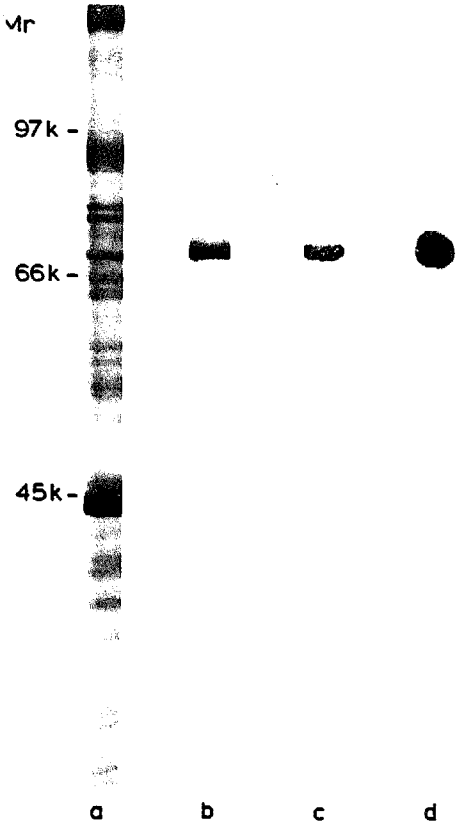

Fig. 3. Characterization of a polyclonal antiserum raised against a [^{14}C]BADG-labeled polypeptide from renal brush-border membranes with an apparent molecular weight of 70000. Protein fraction PI was isolated from brush-border membranes, labeled with [^{14}C]BADG, solubilized with SDS and fractionated by gel chromatography as in Fig. 2. Protein fractions 36 and 37 of Fig. 2 were separated in preparative SDS-polyacrylamide slab gels. After dissection from the unstained gel, the M_r 70000 polypeptide was electroeluted, analyzed by SDS-polyacrylamide gel electrophoresis and used for immunization of rabbits. In the figure silver staining of separated brush-border membranes (a) and of electroeluted M_r 70000 polypeptide (b) is compared. (c) shows an autoradiogram of the [^{14}C]BADG-labeled M_r 70000 polypeptide and (d) the reaction of polyclonal antibodies raised against the M_r 70000 polypeptide with brush-border membranes.

stained M_r 70000 polypeptide was electroeluted and used either to raise polyclonal antibodies or for digestion with V8 proteinase. For trypsin digestion the M_r 70000 polypeptide was dissected from Coomassie-stained SDS-polyacrylamide gels and the proteolyis was performed in gel slices.

Immunohistochemical localization of the catalytic H^+-ATPase subunit in kidney cortex

Fig. 3 shows that the electroeluted [^{14}C]BADG-labeled M_r 70000 polypeptide runs as a single band in SDS-polyacrylamide gels. When a rabbit was immunized with this polypeptide an antiserum was obtained which bound selectively to a M_r 70000 polypeptide from brush-border membranes (Fig. 3d). With this antiserum an immuno-histochemical staining pattern was observed in rat kidney cortex which is typical for the localization of V-ATPases [3]. Thus, the antibodies reacted mainly with intercalated cells of cortical collecting ducts and showed some reaction with proximal tubules (Fig. 4C). In the proximal tubules the antibodies bound to brush-border membranes and subapical vesicles and did not react with basal-lateral membranes (Figs. 4A,B). In the collecting ducts the antibodies reacted strongly with subapical vesicles of intercalated A-cells (Fig. 4D) and showed some reaction with subapical vesicles and basal membranes of intercalated B-cells (Fig. 4E). These data suggested that the isolated BADG-labeled M_r 70000 polypeptide is the catalytic subunit of the H^+-ATPase from brush-border membranes of renal proximal tubules and that this

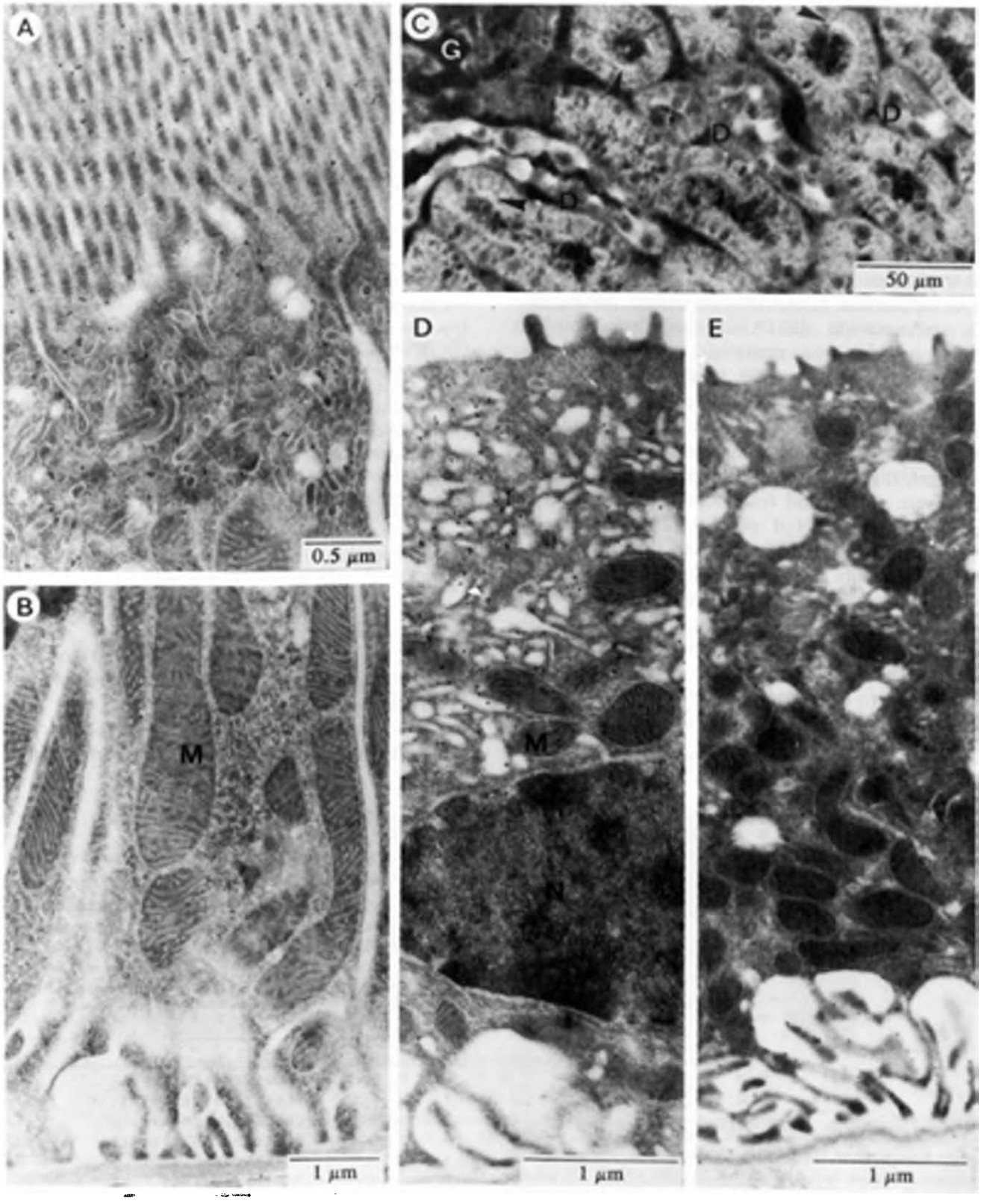

Fig. 4. Immunostaining of rat kidney cortex with an antiserum directed against the [^{14}C]BADG-labeled M_r 70 000 polypeptide from porcine renal brush-border membranes. The antiserum described in Fig. 3 was used for the immune reactions. For light microscopy (C) fixed 5-μm sections were reacted with the antiserum and visualized by an FITC-labeled secondary antibody. For electron microscopy (A,B,D,E) plastic ultrathin sections were reacted with the antiserum and visualized with a gold-coupled secondary antibody. Panel C shows a section through the outer cortex. The arrowheads indicate antibody labeling of the brush-border membrane in proximal tubules. In Panels A and B the apical and basal parts of an epithelial cell from the proximal convoluted tubule are shown, respectively. Panels D and E show different types of intercalated cells from collecting ducts (D, cell type A; E, cell type B). G, glomerulum; D, collecting duct; N, nucleus; M, mitochondrium.

subunit is homologous or identical to the catalytic subunit of the H^+-ATPase(s) from collecting ducts.

Proteolytic digestion and partial sequencing of the catalytic subunit of H^+-ATPase

50 μg of the electro-eluted M_r 70000 polypeptide was treated for 16 h (37°C) with V8 proteinase, separated by SDS-polyacrylamide gel electrophoresis and transferred to a poly(vinylidene difluoride) membrane. After staining with Amido black, five proteolytic peptides could be distinguished (Fig. 5). These peptides were dissected from the membrane and sequenced. Partial sequences from the five peptides are shown in Table II (sequences 1,3,4,8,10). To obtain further sequence information BADG-labeled brush-border membrane proteins were separated by gel chromatography (see Fig. 2) and fractions 36 and 37 containing 2 mg of protein were separated by preparative SDS-polyacrylamide gel electrophoresis. After short staining with Coomassie the M_r 70000 polypeptide was dissected from the gel, digested with trypsin and the proteolytic peptides were eluted and fractionated by HPLC (C_{18} column, gradient of H_2O plus 0.1%(v/v) TFA to 80%(v/v) acetonitrile). Fig. 6 shows the absorbance and the radioactivity distribution of the obtained fractions. From several peaks partial amino acid sequences were obtained (Table II, sequences 2, 5, 7, 9, 11, 13). Fig. 6 shows that significant amounts of BADG were associated with four different peaks. Since these peaks did not appear to be homogeneous they were rechromatographed on the C_{18} column by applying a gradient of 25 mM sodium phosphate (pH 5) to 50%(v/v) acetonitrile in 25 mM sodium phosphate (pH 5). After rechromatography partial sequences were obtained from two radioactive peaks (Table II, sequences 6, 12). Trying to identify the amino acids which are modified

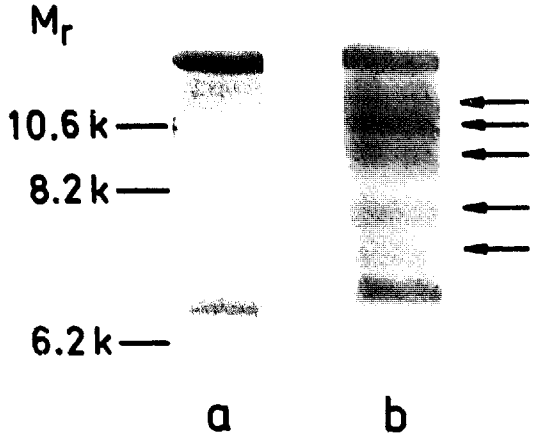

Fig. 5. Proteolytic digestion of the [^{14}C]BADG-labeled M_r 70000 polypeptide from porcine kidney with V8 proteinase. 300 μl of 100 mM ammonium acetate (pH 4.0) without (a) and with (b) 50 μg of the electroeluted M_r 70000 polypeptide shown in Fig. 3 were incubated 16 h (37°C) with 300 μg of V8 proteinase. The samples were concentrated and applied on SDS gels [34]. The separated polypeptides were electrically transferred to a poly(vinylidene difluoride) membrane and stained with Amido black. Five proteolytic fragments of the M_r 70000 polypeptide (see arrows) were dissected for amino acid sequencing. On the left the relative molecular weights of three marker proteins are indicated.

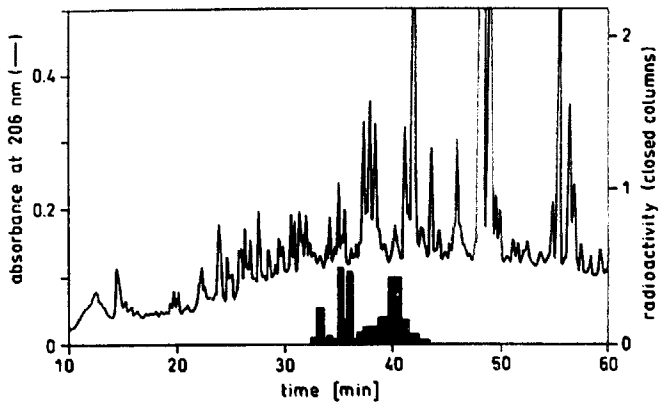

Fig. 6. Fractionation of tryptic digestion products of the [^{14}C]BADG-labeled M_r 70000 polypeptide by reversed phase HPLC. Fractions 36 and 37 from Fig. 2 containing partially purified [^{14}C]BADG-labeled M_r 70000 polypeptide were concentrated and 0.5 mg of protein was subjected to preparative SDS-polyacrylamide gel electrophoresis. The gel was stained and a gel slice containing the M_r 70000 polypeptide was dissected and treated with trypsin. The proteolytic splitting products were eluted from the gels and fractionated by reversed phase chromatography on a C_{18} column. The figure shows an absorbance profile and the radioactivity distribution of a typical separation experiment. Symmetrical slim peaks were selected for amino acid sequencing. Since the four fractions with significant amounts of radioactivity (given in cpm × 10^3) did not appear to be homogeneous they were applied for rechromatography as described in Materials and Methods.

TABLE II

Amino acid sequences of the catalytic H^+-ATPase from brush-border membranes of proximal tubules from porcine kidney

The catalytic H^+-ATPase subunit was labeled, isolated and digested and the proteolytic fragments were purified and sequenced as described in Materials and Methods. Peptides 1, 3, 4, 8, 10 were obtained after digesting of the catalytic subunit with V8 proteinase whereas peptides 2, 5, 6, 7, 9, 11, 12, 13 were obtained after digestion with trypsin.

No.	Sequence
1	TSGVSVGDPVLXFGKPL
2	VGSHITGGDIYGIVNENSLIK
3	LEFEGVKEKFXMVQV
4	KLPANXPLLTGQRVLDALF
5	TVISQSLSK
6	YSNSDVIIYVGXGE
7	ALVANTSNMPVAA
8	ASIYTGITLSEYFRDM
9	LAEMPADSGYPAYLGAR
10	GSVXIVGAVSP
11	ALDEYYDKHFTEFVPL
12	FXPFYK
13	TVGMLSNMIAFYDMA

by [^{14}C]BADG, the released radioactivity after the different cycles of sequencing was measured. For sequence 6 the highest radioactivity was obtained at position 12 where no amino acid could be identified. The radioactivity distribution obtained for sequence 12 suggests that the non-identified amino acid in position 2 was labeled with [^{14}C]BADG. Since the DNA sequencing reported below (see Fig. 7) showed that cysteine residues are localized in position 12 of sequence 6 and in position 2 of sequence 12 (see Fig. 8), it can be assumed that these two cysteines have been modified with the SH-group reagent BADG.

Cloning of the catalytic subunit of H$^+$-ATPase

The cDNA of the catalytic H$^+$-ATPases subunit from porcine kidney cortex was cloned by PCR as described in Materials and Methods. Two overlapping cDNA-fragments (Fig. 7, nucleotides 511–1133 and 1108–1671) were obtained by employing information from three proteolytic peptides (see Table II sequences 2, 9, 13). The 5′-part of the cDNA (Fig. 7, nucleotides 1–513) was cloned with the help of nucleotide sequence information from the catalytic H$^+$-ATPase subunit of bovine adrenal medulla which was published during our study [39]. The 3′-part of the clone (Fig. 7, nucleotides 1609–2098) was obtained by employing the RACE-methodology (see Materials and Methods). Fig. 7 shows the complete nucleotide and amino acid sequence information which was obtained from the overlapping cDNA fragments. Since after subcloning of the different PCR-fragments always identical nucleotide sequences were obtained from different clones the presented sequence is supposed to be the main cDNA-subtype of the catalytic H$^+$-ATPase subunit in pig kidney cortex. 181 of the 183 amino acid residues which were determined by amino acid sequencing of the proteolytic peptides (Table II, sequences 1–13) were identical to the amino acid sequence derived from the cloned cDNA (Fig. 8). Since both deviations are due to a single-base exchange they may be due to genetic variants of individual pigs or to misincorporation of bases during the PCR-reaction rather than to different subtypes of the catalytic H$^+$-ATPase subunits in pig kidney.

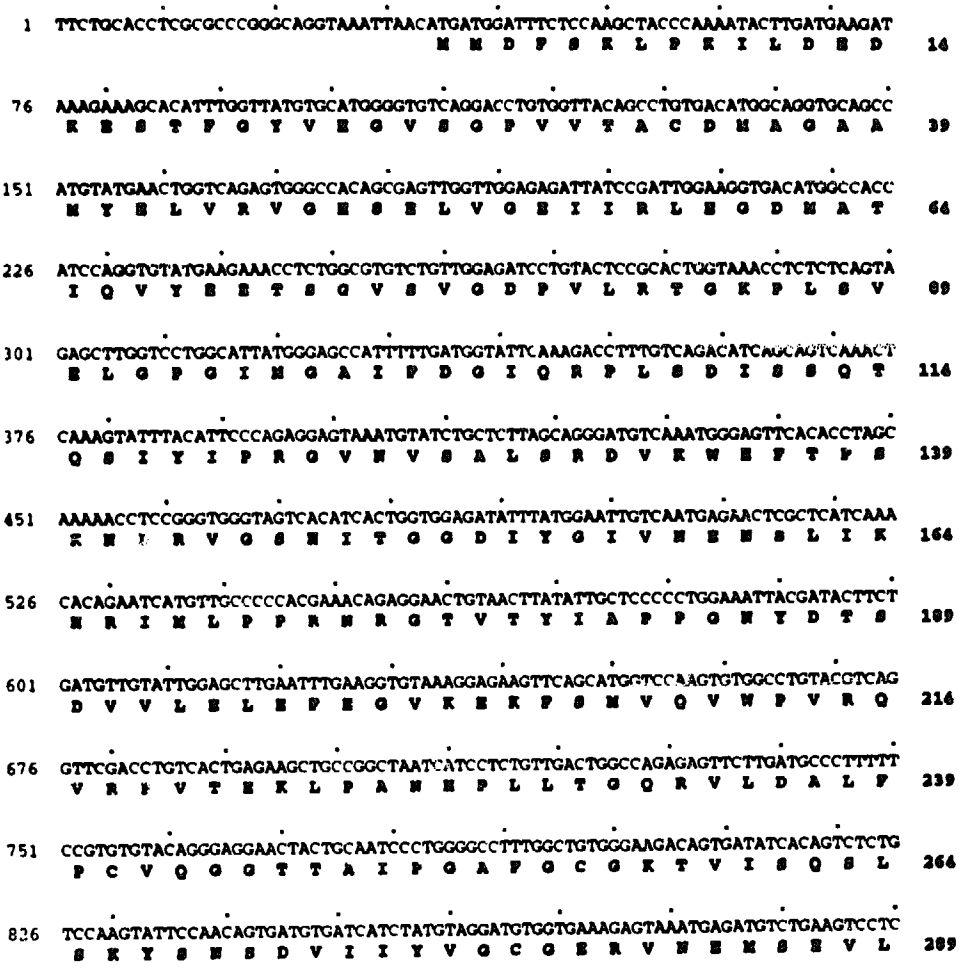

Fig. 7. Nucleotide sequence and derived amino acid sequence of a catalytic H$^+$-ATPase subunit from porcine kidney cortex. The sequence was determined from four overlapping cDNA-fragments obtained by PCR. The numbers on the left and right refer to the nucleotide and amino acid positions, respectively. (Continued on p. 138.)

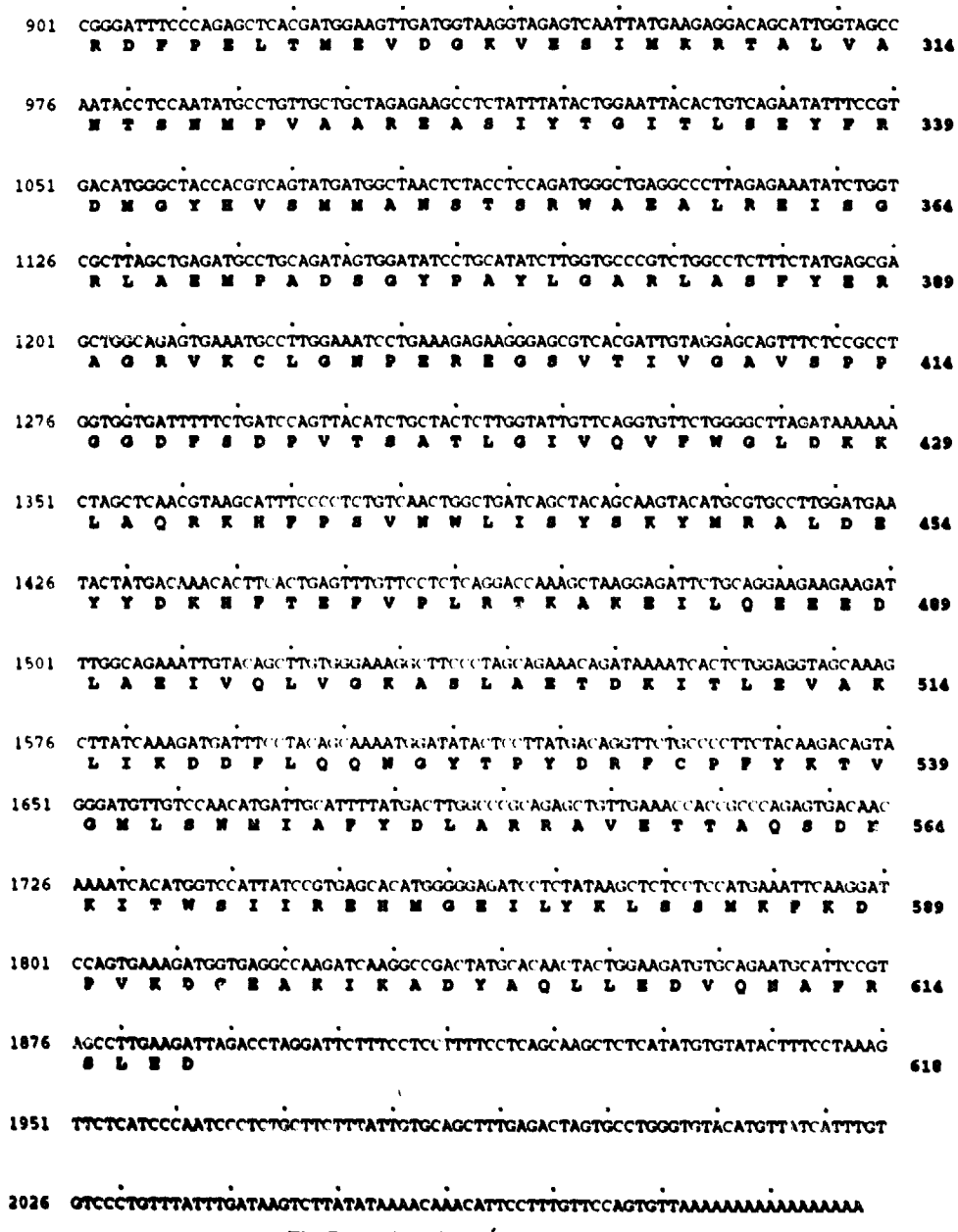

Fig. 7 (continued). For legend see p. 137.

Comparison of amino acid sequences of catalytic H^+-ATPase subunits

The amino acid sequence of the cloned catalytic subunit from porcine kidney described in this paper is compared with the catalytic H^+-ATPase subunits of *Archebacterium sulfolobus acidocaldarius* [44], *Neurospora crassa* [45], *Dauctus carota* [22], bovine adrenal medulla [39] and with partial amino acid sequences which have been isolated from bovine brain [24]. 49% identity was obtained with the catalytic subunit of *Archebacterium sulfolobus acidocaldarius*. *Neurospora crassa* and *Dauctus carota* contained 63% and 69% identical amino acid residues (Fig. 8). Comparison with sequences from the catalytic H^+-ATPase subunit of

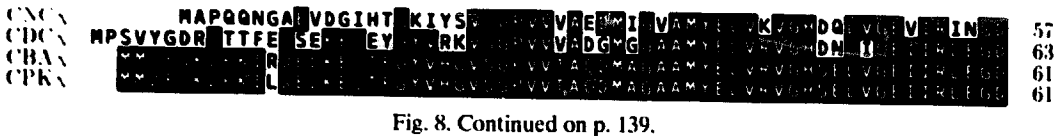

Fig. 8. Continued on p. 139.

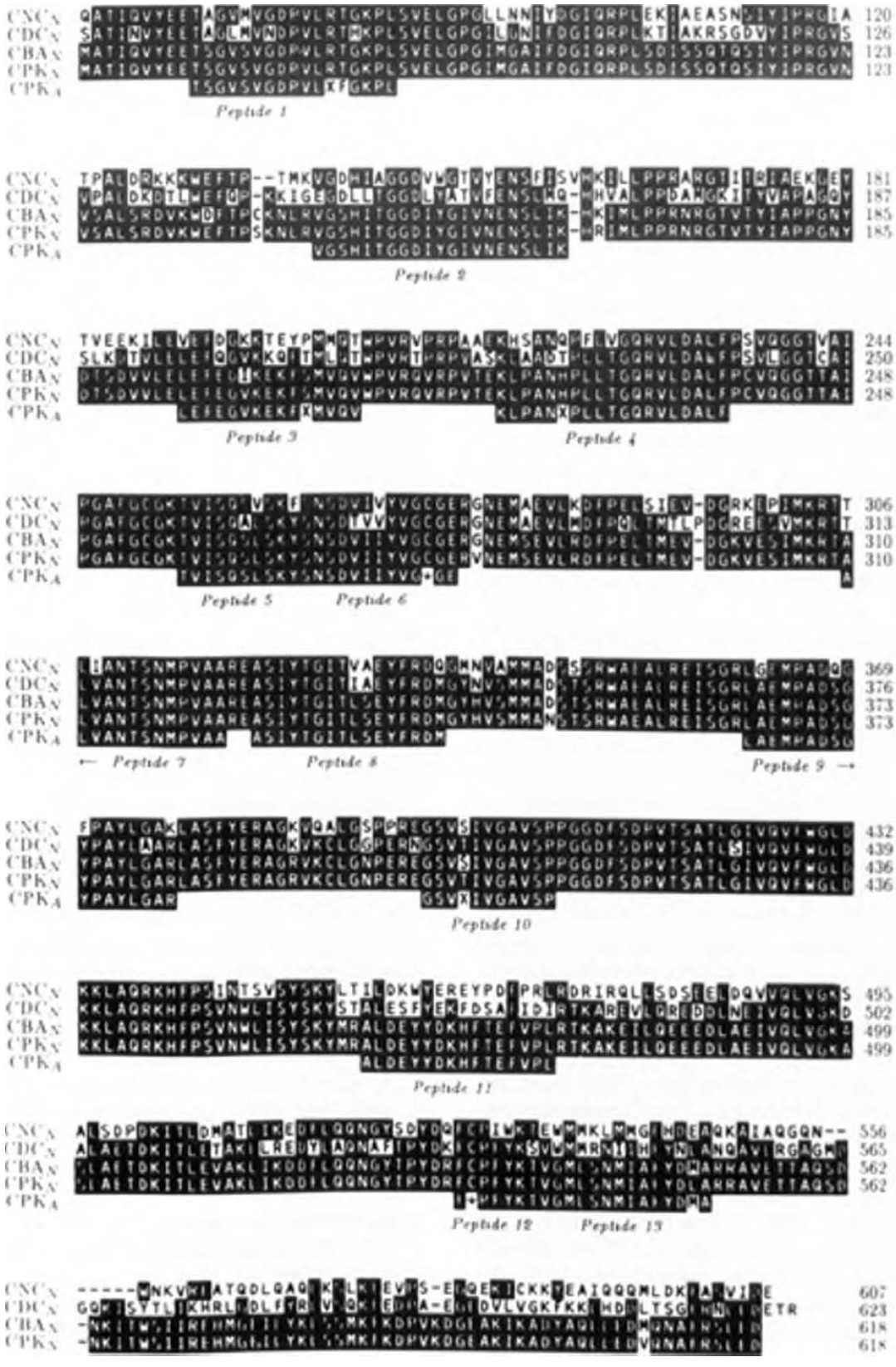

Fig. 8. Comparison of sequences from catalytic subunits of V-ATPases. The following sequences are compared: CNC_N, sequence of the catalytic H^+-ATPase subunit of *Neurospora crassa* derived from cloned cDNA [45]; CDC_N, sequence of the catalytic H^+-ATPase subunit of *Daucus carota* derived from cloned cDNA [22]; CBA_N, sequence of the catalytic H^+-ATPase subunit from bovine adrenal medulla derived from cloned cDNA [39]; CPK_N, sequence of the catalytic H^+-ATPase subunit from porcine renal cortex derived from cloned cDNA; CPK_A, peptide sequences of the catalytic H^+-ATPase from porcine kidney obtained by amino acid sequencing of the transporter from brush-border membranes of proximal tubules (the sequences are numbered as in Table II). Amino acid positions in which radioactivity was obtained after sequencing of peptides from the [^{14}C]BADG-labeled M_r 70000 polypeptide are indicated by asteriks.

bovine adrenal medulla and clathrin-coated vesicles from bovine brain (137 overlapping amino acids) gave identities of 98% and 94%, respectively.

Discussion

This paper reports amino acid sequences from the catalytic subunit of a V-ATPase which is localized in the brush-border of renal proximal tubules. The catalytic subunit was identified by affinity labeling of purified brush-border membrane vesicles, the labeled protein was purified to homogeneity and partial amino acid sequences were obtained after proteolytic digestion. The brush-border membrane vesicles employed for labeling and purification were isolated by differential centrifugation after Ca^{2+}-precipitation and do not contain significant amounts of membranes from other nephron segments or of basolateral membranes from proximal tubules [27,46]. They also did not contain significant amounts of subapical vesicles which contain V-ATPases with ATP binding sites on the outside since no ATP-dependent proton uptake could be detected in the right-side out oriented vesicle preparation (data not shown). Affinity labeling of the catalytic H^+-ATPase subunit was performed with the SH group reagent BADG which binds selectively to a polypeptide with an apparent molecular weight of 70000. Similar to *N*-ethylmaleimide [10,12,16,42,47] BADG is also an affinity label of the H^+-ATPase. Thus, BADG irreversibly inhibits ATP-dependent proton transport into inside-out oriented brush-border membrane vesicles and the inhibition by BADG is prevented by ATP. The selective modification of only a few amino acids of the catalytic H^+-ATPase subunit by [^{14}C]BADG together with complete inhibition of ATP-driven proton uptake by 50 μM BADG and the complete prevention of this inhibition by ATP, suggests that BADG binds to a protein domain which performs structural changes during ATP driven transport of protons. This protein domain is probably localized around Cys-277 of the catalytic V-ATPase subunit (see Fig. 8) since radioactive labeling by [^{14}C]BADG was observed at Cys-277 and the corresponding amino acids in the homologous sequences of the catalytic β-subunits of F_1-ATPases (Val-188 from thermophilic bacterium PS3 [48], Val-179 in the F_1-ATPase from *Escherichia coli* [49] and Val-183 in the F_1-ATPase from bovine mitochondria [23]), are one (thermophilic bacterium) or twelve amino acids (*Escherichia coli* and bovine mitochondria) distant from a glutamic acid which is selectively modified when the F_1-ATPases are irreversibly inhibited by dicyclohexyl [^{14}C]carbodiimide [50,51]. In the F_1-ATPase from *Escherichia coli*, Glu-185 is essential for structure and assembly of the β-subunit [52]. This amino acid is conserved in the catalytic subunit of the H^+-ATPases (see Glu-283 in Fig. 8) and is five amino acids distant from the BADG modified Cys-277. To verify the role of Cys-277 in the ATP-protectable inhibition of the H^+-ATPase from brush-border membranes and to investigate a possible role of Cys-532 which was also modified by BADG, sequencing of the catalytic H^+-ATPase subunit after BADG labeling in the presence and absence of ATP is required.

Employing mRNA from the outer cortex of porcine kidney a cDNA of catalytic H^+-ATPase subunit was cloned which is supposed to be the main transcript in outer cortex. With two exceptions which may be explained by single-base exchanges the derived amino acid sequence shows identity with the sequences of overlapping proteolytic peptides which were isolated from brush-border membranes. The reported amino acid sequences from porcine outer cortex are highly homologous to the catalytic V-ATPase subunits of archebacteria, plants and fungi (see, e.g., Fig. 8). A comparison of the sequences in this paper with those from bovine brain [24] and bovine adenal medulla [39] shows 94% and 98% identity (Fig. 8). Recently Gluck and co-workers cloned the cDNA of the catalytic H^+-ATPase subunit from bovine kidney medulla and communicated the derived sequence before publication. This bovine sequence has 98% identical amino acids with the catalytic H^+-ATPase subunit from bovine adrenal medulla and 96% identical amino acids with the sequence from pig renal cortex. The differences in primary structure between bovine adrenal medulla, bovine renal medulla and porcine renal cortex are small. The apparently larger structural deviation of the catalytic H^+-ATPase subunit from bovine brain [24] may be artefactural since the sequence information derived from amino acid sequencing of proteolytic peptides is limited (137 amino acids) and most deviations were obtained near the C-termini of the peptides. Thus the data suggest that the catalytic H^+-ATPase subunit is highly conserved in different mammalian species. It exhibits some small but significant organ specificity.

In kidney cortex V-ATPases are primarily localized in collecting ducts and proximal tubules where they occur in subapical vesicles as well as in luminal cell membranes. Since sequence information from the catalytic H^+-ATPase subunit of a defined localization is now available, the question may be approached whether or not the catalytic subunits from different localizations in kidney are identical. The cloning experiments reported in this paper are not sufficient to answer this question. This will be possible when further cDNA sequences from porcine kidney are cloned and identified.

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