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Original article

Characterization of aminopeptidase N from *Torpedo marmorata* kidney

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Summary – A major antigen of the brush border membrane of *Torpedo marmorata* kidney was identified and purified by immunoprecipitation. The sequence of its 18 N terminal amino acids was determined and found to be very similar to that of mammalian aminopeptidase N (EC 3.4.11.2). Indeed aminopeptidase N activity was efficiently immunoprecipitated by monoclonal antibody 180K1. The purified antigen gives a broad band at 180 kDa after SDS-gel electrophoresis, which, after treatment by endoglycosidase F, is converted to a thinner band at 140 kDa. This antigen is therefore heavily glycosylated. Depending on solubilization conditions, both the antigen and peptidase activity were recovered either as a broad peak with a sedimentation coefficient of 18S (2% CHAPS) or as a single peak of 7.8S (1% CHAPS plus 0.2 % $C_{12}E_9$), showing that *Torpedo* aminopeptidase N behaves as an oligomer stabilized by hydrophobic interactions, easily converted into a 160 kDa monomer. The antigen is highly concentrated in the apical membrane of proximal tubule epithelial cells (600 gold particles/μm^2 of brush border membrane) whereas no labeling could be detected in other cell types or in other membranes of the same cells (basolateral membranes, vacuoles or vesicles). Monoclonal antibodies prepared here will be useful tools for further functional and structural studies of *Torpedo* kidney aminopeptidase N.

aminopeptidase N / kidney / *Torpedo marmorata* / antibodies / immunoprecipitation

Introduction

Aminopeptidase N (EC 3.4.11.2) is an abundant membrane bound peptidase of kidney and intestinal microvilli (for reviews, see [7, 23]). It hydrolyses the N-terminal amino acid of short peptides, with preference for amino acids with uncharged side chains [23]. While it completes digestion of oligopeptides in the intestine [20], its function in kidney brush border membrane remains unclear. This enzyme has also been reported in the plasma membrane of various cell types like hepatocytes [1, 25] or myeloid cells [11].

The complete amino acid sequences of human intestinal [21] and rat kidney [28] aminopeptidases N have been determined. Northern blot analysis revealed the existence of two human RNA transcripts, both encoding for the same protein but under the control of different promoters. However, intestinal and kidney epithelial cells express the same mRNAs, which are shorter than the transcripts of myeloid or fibroblastic cells [24].

Aminopeptidase N is composed of a single type of subunit, a 140-kDa glycosylated polypeptide (for reviews, see [7, 23]). This subunit possesses a single transmembrane domain near its N terminus while the major part of the enzyme is ectocellular, including the catalytic sites [7, 21, 28].

In the present work, we have characterized the aminopeptidase N from *Torpedo marmorata* kidney and found it to be very similar to the mammalian enzyme.

* Correspondence and reprints

Materials and methods

Membrane preparation

Torpedo marmorata were provided by the marine station of Arcachon (France). Crude fractions of kidney membranes were prepared from pooled whole kidneys which had been stored at –80°C for several days. The tissue was homogenized (10% w/v) in buffer A (100 mM NaCl, 20 mM Na phosphate buffer, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, final pH 7.2) to which 0.5 mM PMSF was added. The homogenate was centrifuged at 500 g_{max} for 10 min. Membranes were pelleted (40 000 g_{max} for 90 min) from the low speed supernatant.

Preparation of monoclonal antibodies

BALB/c mice were immunized by three intraperitoneal injections (at 15-day intervals) of kidney membrane proteins (about 50 μg emulsified proteins in Freund's adjuvant). Three days after a final booster intraperitoneal injection (without adjuvant), immunized spleen cells were fused with the non-secreting myeloma clone P3 × 63-Ag8. 653 cells using polyethylene glycol as the fusing agent [4, 9]. Hybrids were selected in hypoxanthine, aminopterin and thymidine medium and supernatants from the culture wells containing hybrid cells were tested for the presence of antibodies binding to the apical membrane of tubular epithelial cells on *Torpedo* kidney frozen sections. 42 clones were selected, some of which were cloned by limiting dilution.

Immunoprecipitation

Crude kidney membranes (2 mg protein/ml) were solubilized in 1% CHAPS and 0.2% $C_{12}E_9$ at 4°C for 90 min and non-solubilized material was pelleted (160 000 g_{max} for 60 min). For screening experiments, proteins (about 1 mg/ml) were biotinylated using Immunopure NHS-LC-Biotin (Pierce, 100 μg/mg

protein) for 2 h, at 4°C. The reaction was stopped by addition of glycine (150 mM final concentration). Then, immunoprecipitation was performed in one step: 0.5 ml of solubilized proteins (1 mg protein/ml), 1 ml of culture supernatant containing monoclonal antibodies, 0.5 ml of a suspension of protein A Sepharose-4B (Pharmacia, 10 mg dry powder/ml of 200 mM NaCl, 20 mM Tris buffer pH 7.4) and 30 μl of antimouse IgG antibodies raised in rabbit (5 mg Ig/ml, Biosys)were mixed and incubated overnight at 4°C. Beads were recovered by low speed centrifugation (800 g for 5 min) and washed extensively. Bound proteins were eluted by resuspension of the beads in 100 μl sample buffer (5% SDS, 10% β-mercaptoethanol, 1 M sucrose, 50 mM Tris buffer (pH 6.8), 1 mM bromophenol blue), and boiled for 1 min.

Endoglycosidase treatment

Proteins were immunoadsorbed as above and eluted from the Protein A beads in 100 μl SDS sample buffer. They were then diluted in 10 volumes of 250 mM Na acetate buffer, 20 mM EDTA, 10 mM β-mercaptoethanol, 6 mg/ml CHAPS, final pH 6.5 and incubated at 37°C for 18 h with or without 0.5 U endoglycosidase F/N-glycosidase F (Boehringer). After concentration under vacuum, samples were resuspended in SDS sample buffer and submitted to gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed in 8–18% linear acrylamide gradient gels according to [10]. Proteins in acrylamide gels were either stained with Coomassie blue, or electrotransferred onto nitrocellulose as described by [26]. Biotinylated proteins on the blots were indirectly visualized using streptavidin-biotinylated peroxidase complex (Amersham). The peroxidase substrates were H_2O_2 and diaminobenzidine.

N-terminal amino acid sequencing

Immunoprecipitation was performed as above but starting from 18 mg solubilized kidney membrane proteins, and using 30 ml of culture supernatant (mAb 180K2), 300 μl of rabbit antibody to mouse Ig (50 mg Ig/ml, Biosys), and 9 ml of a suspension of Protein A Sepharose-4B beads (10 mg dry powder/ml). Eluted proteins were dialysed against 10 1 0.05% SDS (overnight) and 10 1 0.025% SDS for 4 h (Spectrapor 2), and concentrated to 50 μl under vacuum.

Electrophoresis was performed as above except that 0.1 mM thioglycolate was added to the migration buffer and that a prerun (90 min at 30 mA) was performed [2].

Proteins bands were transferred onto 'Problott' membranes (Applied Biosystems) overnight at 30 V in transfer buffer (50 mM boric acid, 50 mM Tris-base). The 180 kDa band was stained by amidoschwartz and cut out.

Electroblotted proteins were sequenced by Dr JP Le Caer (Institut Alfred Fessard, CNRS, 91198 Gif-sur-Yvette) in a 470 A gaz-liquid protein sequencer (ABI) using the Problott cartridge. Phenylthiohydantoin amino acids were analysed on a 120A PTH analyser (ABI, Foster City, CA).

Velocity sedimentation in continuous sucrose gradients

Kidney membrane proteins (2 mg protein/ml) were solubilized for 1 h at 4°C in one of the following detergent-containing buffers:

1) 100 mM NaCl, 20 mM phosphate buffer, 0.5 mM $MgCl_2$, 10% glycerol, 2% CHAPS; pH 7.4;

2)100 mM NaCl, 20 mM phosphate buffer, 1 mM EDTA, 10% glycerol, 2% CHAPS; pH 7.4;

3) 100 mM NaCl, 20 mM phosphate buffer, 0.5 mM $MgCl_2$, 10% glycerol, 1% CHAPS, 0.2% $C_{12}E_9$; pH 7.4.

After centrifugation (160 000 g_{max} for 1 h), an aliquot (200 or 400 μl supernatant) of solubilized proteins was layered on top of 11 ml sucrose gradient. We used 5–30% linear sucrose gradients in the solubilization buffer but with a 10-fold lower detergent concentration. Proteins markers: alcohol deshydrogenase (7.4S, Boehringer), catalase (11.3S, Boehringer), and β-galactosidase (16S, Sigma) were solubilized and submitted to centrifugation in parallel linear sucrose gradients.

The 180 kDa antigen content in the fractions was estimated after 1/3 to 1/10 dilution in 200 μl transfer buffer (50 mM Tris, 0.01% Triton X-100, 10% methanol; pH 7.4) and blotting onto nitrocellulose, using a dot-blot apparatus. Dots were probed with mAb 180K1 and antimouse Ig antibodies conjugated to peroxidase (Institut Pasteur Productions). Intensity of the dot staining was quantified using a scanning densitometer (Hoeffer Scientific Instruments) and compared to that of known amounts of solubilized kidney membrane proteins. Enzymatic marker activity was measured as previously described [14, 16].

Aminopeptidase N activity was followed at 405 nm using L-alanine p-nitroanilide (Sigma) as the substrate in a 50 mM Tris phosphate buffer (pH 7.4) [30].

Protein content was determined by the method of Lowry [12].

Morphological experiments

Immunofluorescence

Kidney pieces were fixed immediately after dissection in 3% paraformaldehyde in 300 mM NaCl, 100 mM Na phosphate buffer (pH 7.4) for several hours. They were then incubated in the saline containing increasing sucrose concentrations (up to 20%) and frozen in isopentane cooled on dry ice. Sections (10 μm thick) were mounted on gelatin coated microscope slides and stored at –80°C. Primary antibodies (or culture supernatants) were diluted in 500 mM NaCl, 20 mM Tris buffer (pH 7.4), 0.1% Tween 20 and 1% bovine serum albumin (BSA) and allowed to bind overnight. Bound antibodies were visualized using fluorescein conjugated antimouse Ig antibodies (Institut Pasteur Productions).

Electron microscopy

Kidney pieces were immersed in 2.5% glutaraldehyde in 300 mM NaCl, 40 mM Na phosphate buffer (pH 7.0), for 3 h at room temperature. Tissue pieces were permeabilized by 0.2% saponin in the same buffered saline for 30 min and preincubated in 0.4 M Tris buffer (pH 7.5) containing 1% BSA for 2 h. Monoclonal antibodies 180K1 and 180K2 (five-fold dilution in Tris buffer-BSA) were allowed to bind for 18 h at 5°C. After several washes (for 6 h) in 0.4 M Tris buffer, bound antibodies were indirectly visualized using antimouse IgG antibodies conjugated to 5 nm gold particles (Amersham, 1/5 dilution). Tissue pieces were washed in 0.4 M cacodylate buffer (pH 7.4), post-fixed in 2.5% glutaraldehyde in 0.4 M cacodylate buffer for 30 min and then in 2% OsO_4 in 0.2% cacodylate buffer for 60 min, dehydrated in ethanol and embedded in Spurr resin (Taab laboratories).

Results

Characterization of a major immunogen of 180 kDa in kidney membranes

By indirect immunofluorescence on frozen kidney sections, 42 clones were selected which produced antibodies that bound to the apical membrane of proximal tubule epithelial cells. Each of these 42 culture supernatants was used to immunoprecipitate kidney membrane proteins that had been solubilized in CHAPS and biotinylated (see *Materials and methods*). The antigens were then identified, after SDS gel electrophoresis and electrotransfer onto nitrocellulose, using streptavidin biotinylated peroxidase complex. Surprisingly, considering the heterogeneity of the kidney membrane fraction, 17 supernatants (out of 42) immunoprecipitated the same 180 kDa antigen. A typical example is illustrated in figure 1. Monoclonal antibody 180K1 immunoprecipitates a single biotinylated antigen (left panel, lane 3) which gives a broad band at 180 kDa. This procedure, in a single step, allows a high enrichment of this anti-

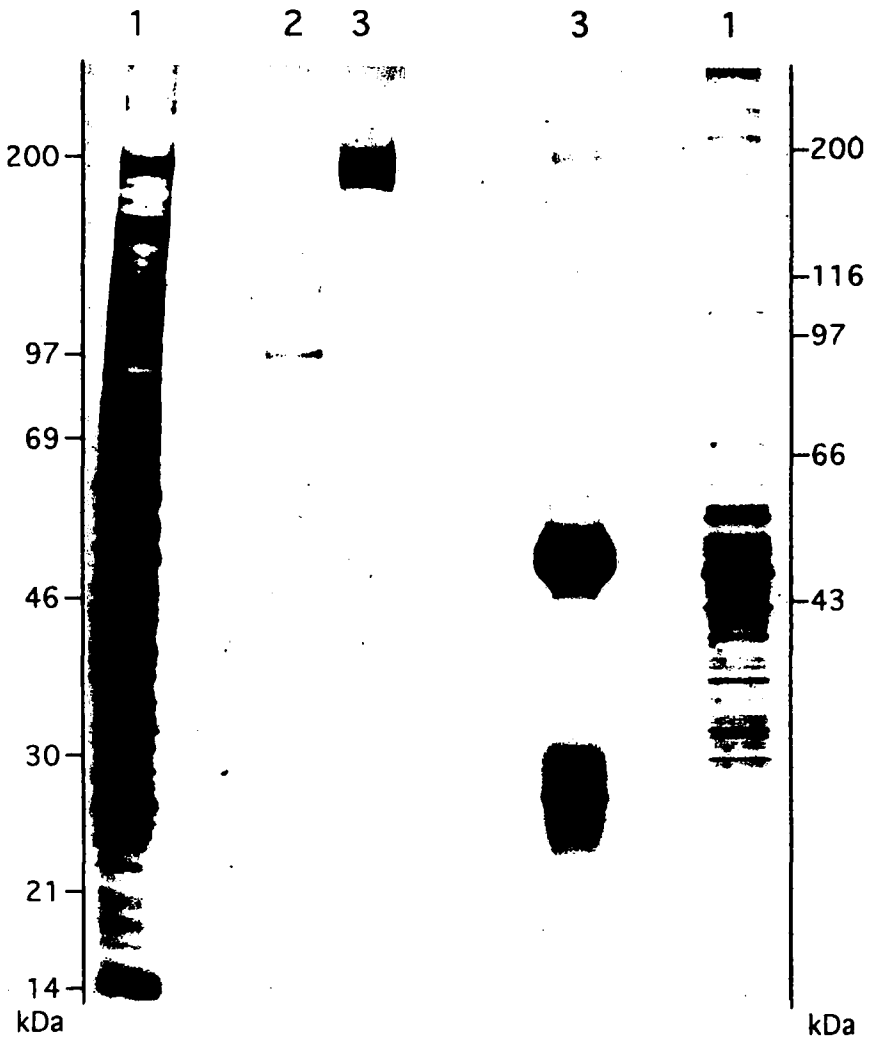

Fig 1. Immunoprecipitation of the 180 kDa antigen. Crude kidney membrane proteins were solubilized and, in some samples (left panel), biotinylated. Solubilized proteins, before (lanes 1) or after immunoprecipitation by monoclonal antibodies 316 (lane 2) or 180 K1 (lanes 3) were submitted to SDS gel electrophoresis. They were either stained in the gels by Coomassie blue (unbiotinylated samples, right panel) or electroblotted onto nitrocellulose and probed with streptavidin-peroxidase (biotinylated samples, left panel).

gen, which is not a major component of the whole biotinylated solubilized kidney membranes (left panel, lane 1). The specificity of the immunoprecipitation is illustrated using another antibody (lane 2) which binds to two lower molecular mass polypeptides. In the absence of biotinylation, the 180 kDa antigen was still immunoprecipitated by mAb 180K1. In this case, it was directly detected in the polyacrylamide gels by Coomassie blue staining (right panel, lane 3), as a broad band of 180 kDa. Biotinylation, therefore, does not modify its electrophoretic behaviour. The Coomassie blue staining of kidney membrane proteins did not reveal an easily detectable band of 180 kDa (right panel, lane 1), showing again that this protein is not a major component of this crude membrane fraction. The bands at 50 and 28 kDa (right panel, lane 3) correspond to the heavy and light immunoglobulin chains.

The 180 kDa antigen behaves as an integral membrane protein since it was not solubilized after treatment of the membranes at high ionic strength (0.5 M NaCl; 0.5 M $MgCl_2$) or at alkaline pH (50 mM Na_2CO_3, pH 11; dot-blot experiments not shown).

In some experiments, when kidney membranes were prepared in the absence of the protease inhibitor PMSF, an additional 150 kDa band was immunoprecipitated by each of the 17 anti-180 kDa antibodies (not shown). This 150 kDa can be very easily removed from membranes by a single washing step. Most probably, it is a proteolytic fragment of the 180 kDa antigen bearing the immunogenic regions but lacking the membrane domains of the protein.

Identification of the 180 kDa antigen

The 180 kDa antigen was immunoprecipitated by monoclonal antibody 180K1, submitted to gel electrophoresis and electroblotted (see *Materials and methods*). The band at 180 kDa was cut out and the N-terminal amino acid sequence was determined using automated Edman degradation. Starting from about 60 pmol of the 180 kDa antigen, 40 pmol PTH-aminoacids were obtained in each of the successive cycles. The 18 N-terminal amino acid sequence of the 180 kDa antigen (table I) turned out to be very similar to the corresponding sequence of aminopeptidase N (EC 3.4.11.2) which has been determined in man [21], rat [28], rabbit [3] and pig [21]. This sequence begins with eight hydrophilic

Table I. N-terminal amino acid sequence of the 180 kDa antigen. For comparison, the N-terminal amino acid sequences of aminopeptidase N from rat kidney [28] and from intestines of man [21], rabbit [31] and pig [21] are shown.

Torpedo kidney	*A K G F Y I S K Q L A V – A G – L A*
Rat kidney	A K G F Y I S K S L G I L G I L L G
Human intestine	A K G F Y I S K S L G I L G I L L G
Rabbit intestine	A K G F Y I S K A L G I L G F X L G
Porc intestine	A K G F Y I S L A L G I A G X L X V

Table II. Immunoprecipitation of aminopeptidase N activity. Solubilized kidney membrane proteins were incubated with monoclonal antibodies (180K1 or 14K4) and protein A-beads. These beads were recovered by low speed centrifugation and washed thoroughly (see *Materials and methods*). Aminopeptidase N activity was estimated in the low speed supernatants (non-retained fractions) or on a suspension of the washed beads (immunoadsorbed fractions). In parallel, the immunoadsorbed proteins were eluted by SDS sample buffer and submitted to SDS gel electrophorésis (see fig 2). Aminopeptidase N activities are expressed in optical density units (OD) per minute at 405 nm for samples deriving from 550 mg of tissue.

mAb	*180K1*	*14K4*
Retained	1.9	0
on protein A	42%	0%
Non-retained	2.6	5.1
	58%	100%

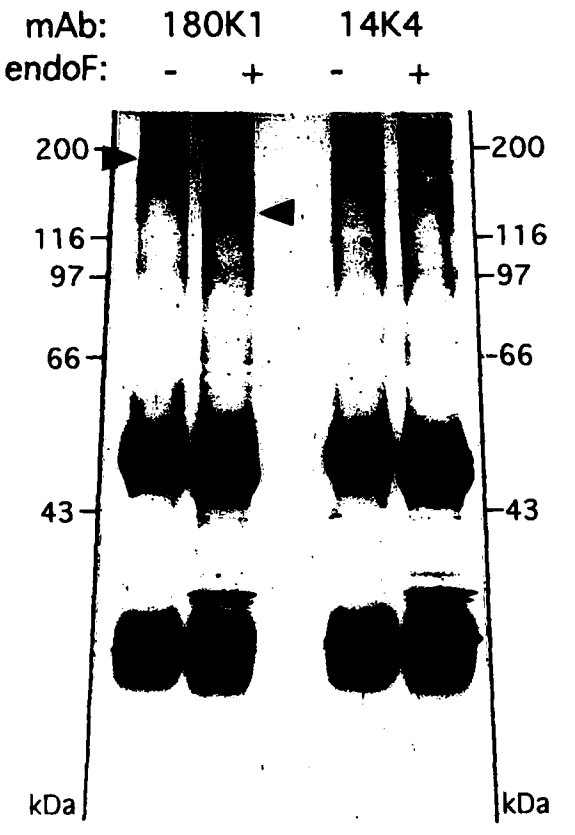

Fig 2. Treatment of immunoprecipitated proteins with glycosidases. Solubilized kidney proteins were immunoprecipitated with monoclonal antibodies 180K1 (lanes 1, 2) or 14K4 (lanes 3, 4). Immunoprecipitated proteins were directly submitted to SDS gel electrophoresis (–) or submitted to endoglycosidase F/N-glycosidase F action (0.5 U at 37°C for 18 h) before electrophoresis. Gels were stained with Coomassie blue.

amino acids followed by hydrophobic residues which are assumed to correspond to the only putative transmembrane domain of aminopeptidase N.

Immunoprecipitation of aminopeptidase N activity

To check whether the 180 kDa antigen was indeed aminopeptidase N, we measured aminopeptidase N activity using alanine p-nitroanilide as the substrate [30].

In the course of immunoprecipitation experiments, enzyme activity was determined both in non-retained samples (incubation mixture after removal of immunoadsorbed proteins by low speed centrifugation of Protein A beads) and in immunoadsorbed protein samples (washed protein A bead suspensions just before SDS elution).

A significant proportion (42%) of peptidase activity was immunoprecipitated in a single step by monoclonal antibody 180K1 (table II). An unrelated monoclonal antibody 14K4 [19] was unable to precipitate any detectable enzyme activity. In the same experiment, the antigen precipitated by antibody 180K1 was visualized as a 180 kDa band after SDS-gel electrophoresis, which was absent from samples incubated with the control antibody 14K4 (fig 2). Proteins immunoabsorbed by antibodies 180K1 or 14K4, and eluted in SDS, were treated by endoglycosidase F (see *Materials and methods*). The 180 kDa band was converted to a lower molecular mass polypeptide, at 140 kDa (fig 2). This demonstrates that *Torpedo* aminopeptidase is heavily glycosylated, deglycosylation reducing its apparent molecular mass by about 20%. Note, for comparison, a small effect of endoglycosidase treatment on the heavy immunoglobulin chain migration (at about 50 kDa) in both 180K1 and 14K4 samples. Bands at 30 kDa most probably correspond to endoglycosidase F (32 kDa molecular mass).

Velocity sedimentation of the 180 kDa antigen and aminopeptidase activity in sucrose gradients

To investigate whether the *Torpedo* kidney aminopeptidase N was a monomer or an oligomer, we studied the velocity sedimentation in sucrose gradients of both the 180 kDa antigen and the aminopeptidase N activity, solubilized in non-denaturing conditions.

Kidney membranes were treated by 1% CHAPS + 0.2% $C_{12}E_9$. Solubilized proteins were sedimented in 5–30% linear sucrose gradients at 40000 rpm for 18 h. Fractions were collected from the bottom of the gradients and their 180 kDa antigen content and peptidase activity were estimated as described in *Materials and methods*. Both antigen and activity were recovered as a single peak, with a sedimentation coefficient of 7.8S (fig 3).

Considering that aminopeptidase N is a hydrophilic membrane protein with a single transmembrane domain [7, 21], we can assume that it binds only a small amount of detergent and behaves as a globular protein in sucrose gra-

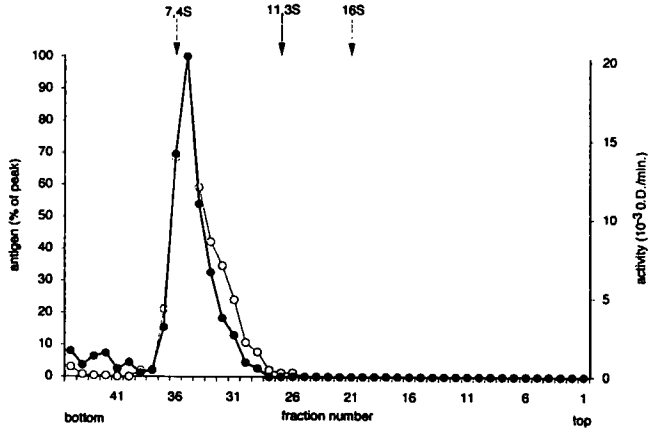

Fig 3. Velocity sedimentation of the 180 kDa antigen and of aminopeptidase N activity solubilized in the presence of $C_{12}E_9$. Crude kidney membranes were solubilized by 1% CHAPS and 0.2% $C_{12}E_9$ in the presence of $MgCl_2$ and centrifuged in 5–30% sucrose gradients (see *Materials and methods*). The 180 kDa antigen content (●—●) and aminopeptidase N activity (○—○) of each fraction was determined. Both migrated in a single peak with a sedimentation coefficient of 7.8S.

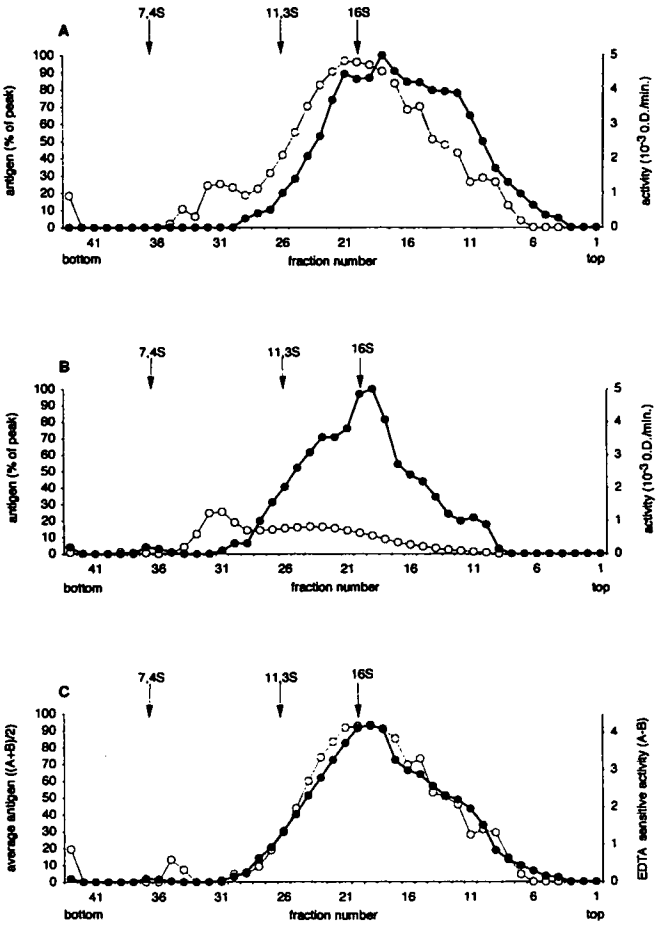

Fig 4. Velocity sedimentation of the 180 kDa antigen and peptidase activity solubilized by CHAPS only. Membranes were solubilized by 2% CHAPS in $MgCl_2$ (**A**) or EDTA (**B**) containing buffers. Sedimentation profiles of the antigen (●—●) and peptidase activity (○—○) in 5–30% sucrose gradients were determined as in figure 3. A broad peak of antigen around 18S was observed both in $MgCl_2$ (**A**) or EDTA (**B**) gradients. Peptidase activity was much lower in the presence of EDTA (**B**) and its distribution differed markedly in both conditions. The EDTA sensitive peptidase activity (**C**) was estimated by difference (**A, B**) and found to comigrate with the antigen (pooled results from **A** and **B**).

dients. Taking the approximation of Martin and Ames [14], this would give an approximate molecular mass of 160 kDa for the 180 kDa antigen, which therefore behaves, in these solubilization conditions, as a monomeric protein.

Then kidney membranes were solubilized by 2% CHAPS alone, in the presence of 0.5 mM $MgCl_2$ (fig 4A) or of 1 mM EDTA (fig 4B). After velocity sedimentation in sucrose gradients containing either $MgCl_2$ or EDTA, a broad peak of antigen was found, with a sedimentation coefficient around 18S. In the presence of $MgCl_2$, the peak of activity was also broad and it did not coincide with that of the antigen (fig 4A): it was shifted to lighter fractions. In addition, a second smaller peak of activity was recovered around 9.5S. In the EDTA containing gradients, the total peptidase activity was much lower than in the $MgCl_2$ conditions. It corresponds to EDTA insensitive peptidase activity. Its distribution (fig 4B) revealed a peak at 9.5S, very similar in amplitude and position to the 9.5S noticed in $MgCl_2$. Some activity was also detected in denser fractions. By difference, it was possible to estimate the distribution of EDTA sensitive peptidase activity, which is similar to that of the antigen (fig 4C).

Aminopeptidase N has been shown to be a zinc metalloprotein [7, 23] and inhibition of its activity by the divalent cations chelator EDTA was not surprising. When solubilized by CHAPS alone, *Torpedo* aminopeptidase behaves as a large size oligomer. This oligomer is dissociated in the presence of 0.2% $C_{12}E_9$, and appears therefore to be stabilized by hydrophobic interactions. The presence of dithiothreitol during solubilisation and centrifugation does not modify the sedimentation profile of *Torpedo* aminopeptidase (data not shown), showing that disulfide bonds are not necessary for its oligomerization, as reported for mammalian aminopeptidase [13].

Localization of the 180 kDa antigen in Torpedo kidney

Torpedo kidney organization has been schematically described by Gérard [5]. A thin tubular segment, with a ciliated epithelium, connects the glomerule to the proximal convoluted tubule, characterized by its large epithelial cells with their typical brush border apical membrane. The distal tubule follows, with a flat epithelium and epithelial cells devoid of flagella or microvilli. In the large field view presented in figure 5 (upper panel), proximal tubule sections are concentrated on the left whereas distal tubule sections are mainly located on the right. Anti-180 kDa antibodies labeled very intensely the apical membrane of proximal tubule epithelial cells. No staining of the basolateral membranes was observed, even at higher magnification (lower panel). Distal tubules and glomerules were not labeled. In sections of boundary segments of the proximal tubule, the staining was restricted to the apex of the larger epithelial cells.

The distribution of the 180 kDa antigen was studied at the subcellular level using monoclonal antibodies 180K1 and 180K2 (figs 6 and 7). These antibodies were chosen because their binding to kidney membranes adsorbed onto

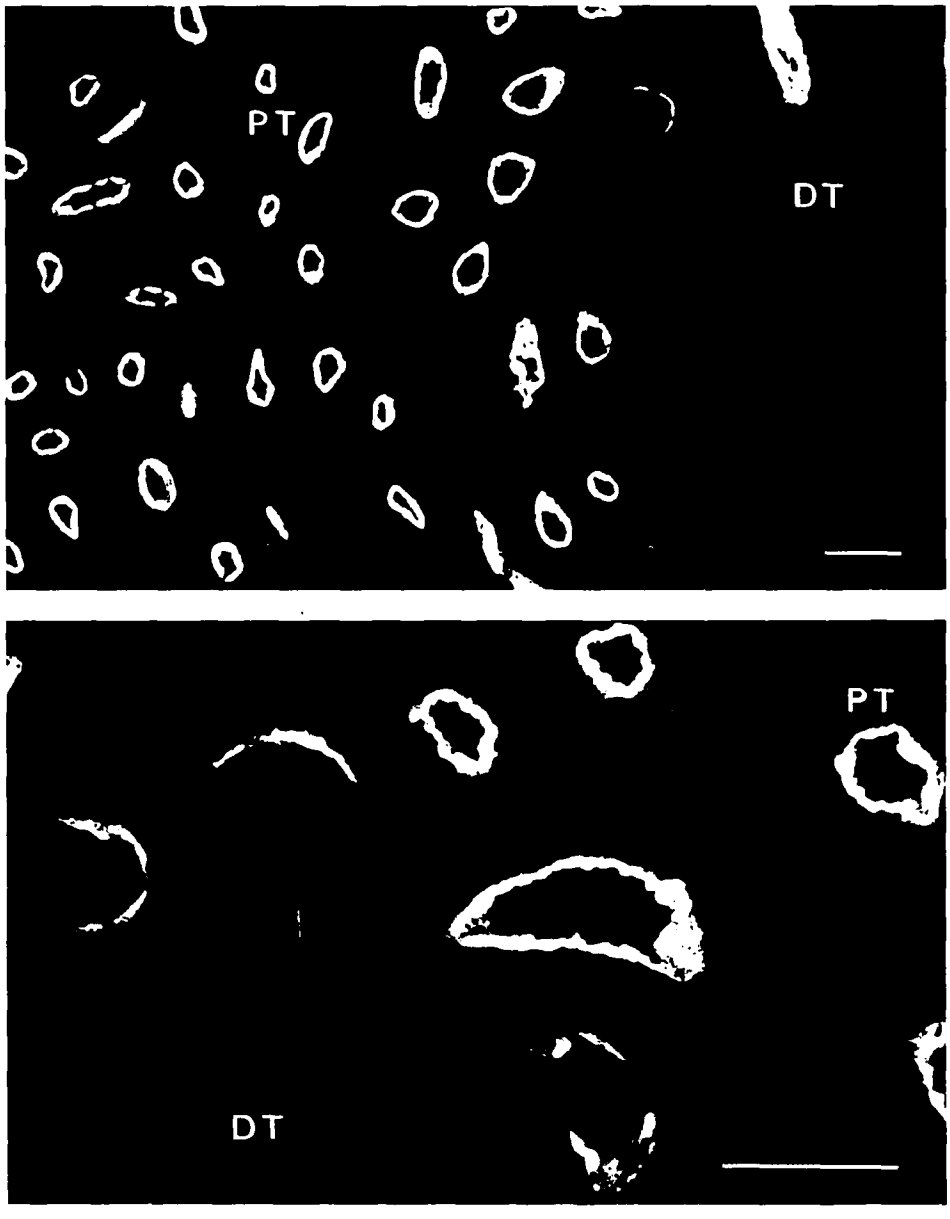

Fig 5. Cellular distribution of the 180 kDa antigen in *Torpedo* kidney. Antibody 180K1 binding in frozen *Torpedo* kidney sections was indirectly visualized using FITC-conjugated antimouse IgG antibodies. The apical membrane of proximal tubule (PT) epithelial cells was heavily stained. No labeling of distal tubules (DT) was detected. Bars = 100 μm.

nitrocellulose was not affected by incubation of the blots in 3% glutaraldehyde (dot blot experiment not shown). Binding of the mAbs 180K1 or 180K2 was indirectly visualized using antimouse IgG antibodies conjugated to 5 nm gold particles. A general view of the apical portion of a proximal tubule epithelial cell is presented in figure 6. The apical membrane covers numerous microvilli, about 0.15 μm large and several microns long. This membrane is homogeneously decorated with numerous gold particles. In contrast, no labelling was associated to the basolateral membrane, or to the numerous vesicular or vacuolar membranes located under the brush border. In higher magnification views (fig 7) the abundance and selectivity of the 180 kDa antigen distribution are demonstrated. The density of gold particles associated to the apical membrane (about 600 particles/μm^2) was estimated on membrane profiles perpendicular to the planes of the section. Gold particles are located extracellularly, at some distance (about 25 nm) from the membrane, except of course on tangential sections (fig 7, top panel).

In some sections of proximal tubules, ciliated cells were found intercalated between epithelial cells (fig 7, lower panel). No gold particles were associated to their apical membrane, nor to basolateral membranes, in contrast to the microvilli plasma membrane of the adjacent epithelial cells.

Discussion

Working on *Torpedo* kidney membranes, we have characterized a major immunogen of the brush border membrane of proximal tubules. In a fusion experiment, we selected hybridoma clones secreting antibodies which bound to these membranes. 40% of these clones produced

Fig 6. Localization of the 180 kDa antigen in kidney brush border membrane. Antibody 180K2 binding in glutaraldehyde fixed *Torpedo* kidney was detected using antimouse IgG conjugated to 5 nm gold particles. A large view of the brush border membrane of proximal tubule epithelial cells is presented. Microvilli are decorated with numerous gold particles, whereas no labeling of the basolateral membranes, vacuoles or vesicles was detected. Bars = 1 μm.

antibodies which precipitated the same 180 kDa antigen, a protein which was only a minor component of kidney membranes. This antigen was highly purified in a single immunoprecipitation step, followed by gel electrophoresis.

This purification allowed us to determine the N-terminal amino acid sequence of the antigen, which turned out to be very similar to that of aminopeptidase N from various mammals including man. The first eight amino acids, which probably correspond to the cytoplasmic domain, are identical in all sequences determined; the following amino acids are hydrophobic and some conservative changes are observed. In addition to the 180 kDa antigen, monoclonal antibody 180K1 immunoprecipitated efficiently aminopeptidase N activity, showing that the 180 kDa antigen carries the activity.

Mammalian forms of aminopeptidase N are heavily glycosylated membrane proteins composed of a single type of subunit (M_r of 160 kDa, detergent form [7]) which possess a single transmembrane domain near its N terminus (see [7, 23] for reviews; [21]). The *Torpedo* protein was not extracted from membranes at high ionic strength or by alkaline (pH 11) treatment, demonstrating that it is an intrinsic membrane protein. It gave a broad band after SDS gel electrophoresis and migrated in these conditions with an apparent molecular mass of 180 kDa. When deglycosylated by endoglycosidase F/N-glycosidase F, the antigen gave a thinner band at 140 kDa. Thus, the *Torpedo* enzyme is also a heavily glycosylated protein; this would explain its high immunogenicity.

When solubilized in 2% CHAPS alone, *Torpedo* aminopeptidase N was recovered in large protein complexes with a sedimentation coefficient around 18S. Dithiothreitol was unable to dissociate these complexes (not shown), demonstrating that disulfide bonds were not necessary for the oligomerization of the enzyme, as previously reported [13]. In

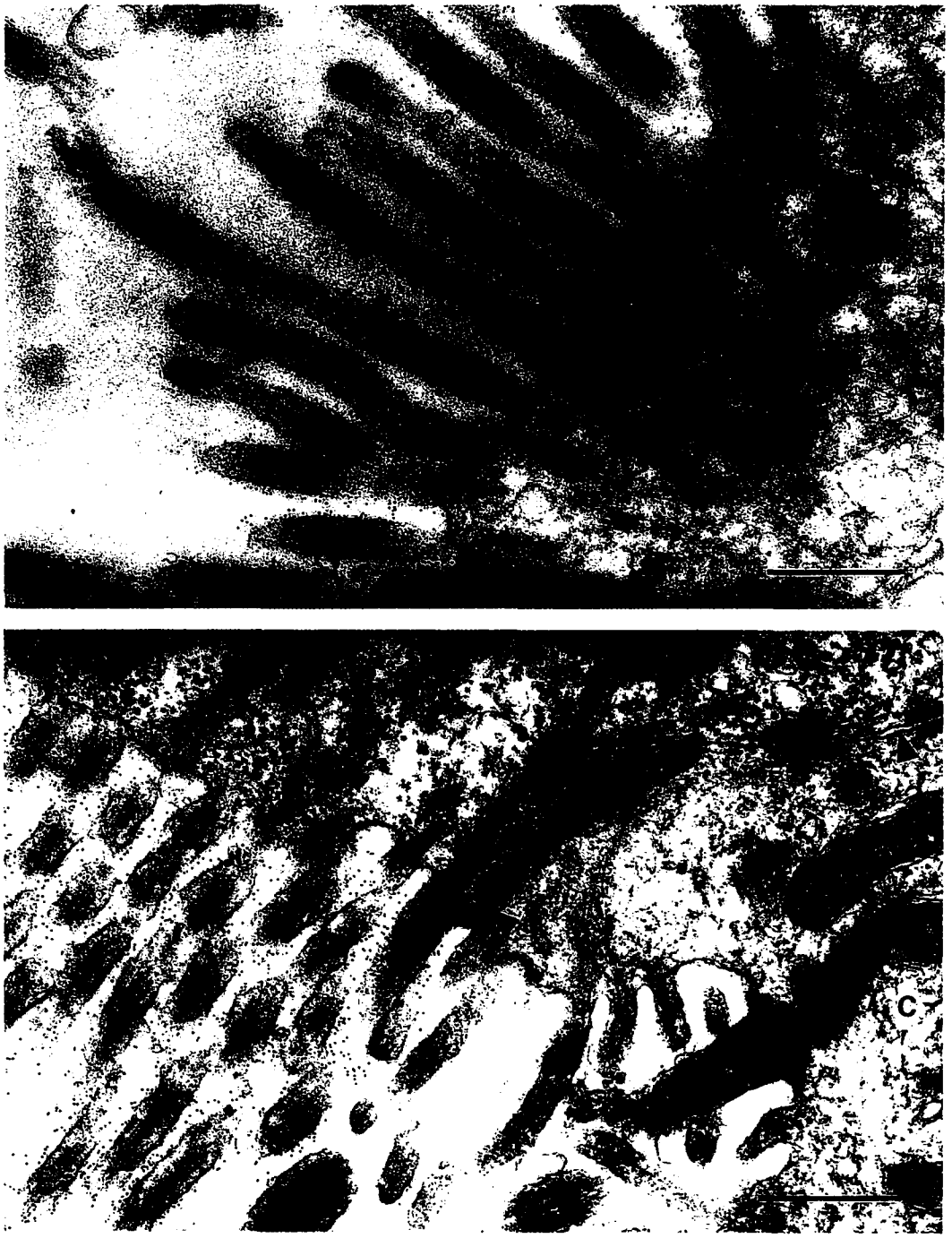

Fig 7. Immunogold labeling of the microvilli membranes. As in figure 4, at higher magnification. Top panel, detail of the brush border membrane; bottom panel; occasionally, ciliated cells (C) are found in between the epithelial cells. The apical membrane of these cells as well as basolateral membranes (▶) are not labeled. Bars = 0.5 μm.

contrast, low concentrations of $C_{12}E_9$ were sufficient to dissociate the oligomers showing that they were stabilized by hydrophobic interactions.

In some experiments (not shown), when proteases were not inhibited by PMSF, a 150 kDa polypeptide was immunoprecipitated in addition to the 180 kDa antigen. This polypeptide, which most probably is a proteolytic fragment of aminopeptidase N, could be eliminated by a simple washing of the crude membranes. This shows that it has lost the transmembrane anchoring domain of the molecule and that it does not remain membrane associated through an interaction with an intact aminopeptidase N monomer, as has been reported for proteolytic digests of porcine [13, 27] and bovine [22] aminopeptidase N.

In *Torpedo* kidney, a high density of the antigen (about 600 gold particles/μm^2) was detected in the brush border membrane in proximal tubule epithelia. No gold particles were associated with the apical membrane of other cells, either ciliated cells in proximal tubules or epithelial cells in distal tubules (not shown). No antigen was detected in

other membranes of proximal tubule epithelial cells, neither basolateral membranes nor intracellular membranes such as vacuoles or vesicles membranes were labeled. Mammalian peptidase N is concentrated in microvillar apical membranes of both kidney proximal tubules and intestinal epithelia. But in enterocytes, enzyme was found associated to purified basolateral membrane fractions, where its specific activity is 20 times lower than that of the brush border membrane fractions [18]. Similarly, in Madin-Darby kidney cells transfected with human aminopeptidase N cDNA, this enzyme was predominantly recovered in the apical membrane but some of it (about 20%) was associated to basolateral membranes [29]. It was therefore surprising that we could not detect any enzyme in basolateral membranes of *Torpedo* kidney, especially considering the high density of gold particles associated with the apical membrane and the absence of background staining.

Sorting of aminopeptidase N has been extensively studied in various cell types: hepatocytes [1], enterocytes [15, 17] and kidney epithelial cells [29]. In Madin-Darby canine kidney cells, aminopeptidase N is directly sorted to the apical plasma membrane [29]. No label was detected in intracellular vesicles in our experiments, suggesting a reduced accessibility of antibodies to these structures. This could also reflect a low turnover of the antigen or possibly that antibodies bind only to mature forms of the enzyme and not to its precursor forms, present in the endoplasmic reticulum and the Golgi apparatus [15, 29].

The major part of mammalian aminopeptidase N, including the catalytic domain, is located on the extracellular surface of the epithelium [7, 23]. This is also the case for the *Torpedo* kidney enzyme. Indeed, epitopes labelled by antibodies 180K1 and 180K2 are exposed in the extracellular medium since gold particles were on the external side of microvilli membranes, located at some distance (about 25 nm) from the membrane. Considering the size of gold particles (5 nm) and that of immunoglobulins G (7 nm), we can estimate that the epitope is about 10 nm outside the plane of the membrane and therefore that a large part of the antigen is localized in the tubular lumen.

In conclusion, we have identified the kidney aminopeptidase N from *Torpedo marmorata*. To our knowledge, it is the first characterization of this enzyme from *Torpedo* and from fish in general. Monoclonal antibodies prepared here will be useful tools for further functional studies since they bind to the solubilized protein without loss of its enzymatic activity. Fine ultrastructural studies will also be possible since these antibodies are able to probe aminopeptidase N in its membrane environment, even after glutaraldehyde fixation of tissues. By its subcellular distribution, its biochemical properties and its N-terminal amino acid sequence, this enzyme closely resembles the mammalian enzymes.

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