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The oligomeric structure of renal aminopeptidase N from bovine brush-border membrane vesicles

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Bovine renal brush-border membrane vesicle aminopeptidase N at various stages of purity was treated with two bifunctional cross-linking agents. A pattern of emergence of higher molecular weight forms was observed. By using a cleavable cross-linker, aminopeptidase N was shown to cross-link both to itself and to its breakdown products as well as to dipeptidyl peptidase IV. Using this technique it was possible to identify three of the breakdown products as 45 kDa, 66 kDa and 95 kDa peptides. N-terminal amino acid sequence analysis was used to define the precise cleavage points for the bovine renal aminopeptidase N breakdown products. The short amino acid sequences obtained show strong sequence similarity with the human intestinal and rat kidney aminopeptidase N.

Introduction

Aminopeptidase N (APN) (EC 3.4.11.2) hydrolyses the N-terminal amino acid from a range of peptides. It constitutes a major protein in intestinal and renal brush borders. Recently APN has been shown to be the major receptor for the enteropathogenic coronavirus TGEV [1] and for human coronavirus 229E [2]. It has also been found to be one of the CD glycoproteins in human myeloid cell plasma membranes [3].

APN is an ectoenzyme anchored to the brush-border membrane by a transmembrane helical region near the N-terminus [4]. A small region of the amino terminus protrudes into the cytoplasm. The main part of the protein, including the active site faces outside the cell and is connected to the transmembrane region by a short 'stalk' or junctional section. The proteinase papain (which has been used to solubilise APN) cleaves the enzyme at the end of the junction on the outer membrane surface [5]. The transmembrane stalk segment remains attached to the membrane while the rest of the molecule is detached with no loss of enzyme activity.

The molecular size of intestinal microvillar APN and its postulated multimeric and subunit forms have been extensively studied (see Refs. 6 and 7 for reviews). Human and rat intestinal APN occurs as a symmetrical dimer of 130 kDa and rabbit APN occurs as a monomer [6]. SDS gel electrophoresis of pig APN shows at least three separate subunits [8,9], and a number of different structures for this enzyme have been postulated [10]. Some of the subunit forms of the pig enzyme are due to proteolysis in vivo or in vitro. In contrast only limited studies have been carried out on APN from renal brush borders. In an early report pig kidney APN was reported to form high molecular weight aggregates which dissociated into a 280 kDa which further dissociated to yield a subunit of 140 kDa [11].

The renal enzyme has some particular points of interest. Unlike the intestinal enzyme which hydrolyses dietary peptides, the renal enzyme has no established physiological function although it is present in great abundance. We have recently presented evidence to suggest that bovine renal APN plays a role in Na⁺-dependent amino acid transport in bovine brush-border membrane vesicles (BBMV) [12]. A number of other studies have recently implicated similar proteins (i.e., polypeptides with only one transmembrane region) in the regulation of Na⁺-independent amino acid transport [13,14]. A model for such a protein acting as some sort of transport system would be more feasible if the proteins had more than one transmembrane region. This could be achieved by association of the protein into oligomeric structures. A number of studies suggest

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Abbreviations: APN, aminopeptidase N; BBMV, brush-border membrane vesicles; DMS, dimethyl suberimidate; DTBP, dimethyl 3,3'-dithiobis(propionimidate); DPP IV, dipeptidyl peptidase IV.

that intestinal APN can form dimers and other structures with its breakdown products, but it is not known if this is also the case in renal membranes [9,10].

BBMV when separated on polyacrylamide gels yield a complex mixture of polypeptides. Identification of any of the constituents can prove problematic. Even though APN is a major constituent (about 5%) of bovine BBMV, discernment of any of its postulated subunits or breakdown products has proved to be difficult. The technique of covalent cross-linking with bifunctional agents has been used in a number of cases to determine oligomeric structures of specific proteins within complex mixtures including intestinal APN [10,9]. In this paper the techniques of cross-linking, immunoaffinity column purification and N-terminal sequence analysis are used to elucidate the oligomeric structure of bovine renal APN.

Methods

Preparation and fractionation of bovine BBMV. Vesicles were isolated from fresh bovine kidney by a modification of the MgCl_2 precipitation method [16] as described in detail elsewhere [17] except that mannitol was replaced by an equal concentration of sucrose in the homogenisation medium. The vesicles were washed and suspended in a medium containing 0.25 M sucrose, 10 mM K^+ Hepes and 0.2 mM CaCl_2 at pH 7.4 and were rapidly frozen in liquid nitrogen and kept at -20°C until used.

Cross-linking with dimethyl suberimidate (DMS) and dimethyl 3,3'-dithiobis(propionimidate) (DTBP). This was a modification of the method of Labbé et al. [18]. BBMV were centrifuged at $100\,000 \times g$ for 30 min at 4°C and the pellets resuspended in 100 mM triethanolamine-HCl (pH 8.3) at $2 \mu\text{g}$ protein/ μl . Other protein samples were dialysed to remove Tris and resuspended in the above buffer. The cross-linker was made up in triethanolamine buffer at 30 mg/ml and added to the protein mixture to give a final concentration of 0.6 mM. After incubation at room temperature for the appropriate time the reaction was quenched by adding glycine to a final concentration of 100 mM glycine at pH 8. Samples were frozen at -20°C until analysed.

Immunoaffinity chromatography. The monoclonal antibody FD19 was prepared as described in a previous paper [19] and was covalently attached to cyanogen bromide (CNBr)-activated Sepharose. Brush-border membranes were solubilised in the detergent MEGA-10 [20] in 20 mM Tris-HCl (pH 7.5) (3 mg detergent/mg protein) and the solution was clarified by centrifugation at $100\,000 \times g$ for 30 min at 4°C . Aliquots of the suspension were passed down a column of FD19 attached to Sepharose. After washing with the same medium, the column was eluted with 50 mM dieth-

ylamine pH 11 and samples were collected in 0.5 M Tris-HCl (pH 7.4) (to give 50 mM Tris-HCl final concentration). Fractions containing protein were pooled and concentrated using Amicon micro-concentrators.

Concanavalin A column chromatography. This was performed as described by Doyle and McGivan [19].

Papain digestion. BBMV (10 mg protein in 1 ml) were digested with $2 \mu\text{l}$ of 25 mg/ml papain in 50 mM Tris-HCl (pH 7.5) for 10 min at room temperature. To remove solubilised APN the suspension was centrifuged at $100\,000 \times g$ for 30 min at 4°C and the pellet was subsequently dissolved in 50 mM Tris-HCl/0.5% MEGA-10 (pH 7.4).

Preparation of samples for N-terminal sequence analysis. Purified protein samples (typically $10 \mu\text{g}$) prepared as described above were separated by SDS-PAGE and Electroblotted (LKB Semi-Dry Blotter) on to Pro-Blott membrane (Applied Biosystems). The desired bands were cut out and subjected to automated amino acid sequence analysis on a 477A/120A Protein Sequencer (Applied Biosystems) with the Blott cartridge using standard cycles.

Assay of aminopeptidase activity. APN activity was assayed at 37°C in a medium containing 50 mM Tris-HCl at pH 7.4 together with 2 mM L-leucine-*p*-nitroanilide. The initial rate of increase of absorption at 410 nm was monitored continuously.

Gels and Western blotting. SDS-PAGE was performed as described by Laemmli [21]. Western blotting was performed by the method of Towbin et al. [22] except that the nitrocellulose was blocked with phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4) containing 5% skimmed milk powder plus 0.2% Tween 20, and washed with phosphate buffered saline containing 0.2% Tween-20. Determinations of molecular weights were made from gels relative to known standards (Bio-Rad Broad-range for gels and Sigma Prestained for blots).

Scanning of gels and blots. Dried gels and blots were scanned using a Chromoscan 3 (Joyce Loebel, Tyne and Wear, UK) at 0.5 Absorbance full scale. Blots were scanned on the reflectance setting at the same sensitivity.

Protein was assayed as described by Bradford [23].

DMS and DTBP were purchased from Sigma (Poole, Dorset). MEGA-10 was synthesised as described by Hildreth [20]. Peroxidase-linked goat anti-mouse IgG was purchased from Bio-Rad (Richmond, CA).

Results

Cross-linking of proteins in BBMV with the bifunctional reagent DMS

DMS is a non-cleavable cross-linker and was used initially to avoid ambiguities due to partial hydrolysis products that may result from the use of cleavable

cross-linkers during SDS-PAGE analysis. In preliminary experiments, intact BBMV were incubated at room temperature in the presence and absence of the DMS as described in Methods. Aliquots were removed at various time points and subjected to SDS-PAGE analysis. Incubation of BBMV at room temperature in the absence of cross-linker led to a progressive decrease in the amount of high molecular weight material and a concomitant increase in lower molecular weight degradation products. In the presence of DMS, protein bands of higher molecular weight accumulated at the expense of lower molecular weight material. This indicates that DMS is able to cross-link proteins in intact BBMV.

Fig. 1 presents a Western blot of DMS treated and untreated BBMV blotted with the monoclonal antibody FD19. This antibody reacts with the 130–150 kDa aminopeptidase N monomer of bovine renal brush borders (described in Refs. 19 and 12). It also reacts with a 260 kDa band and a 45 kDa band in the BBMV preparation. After 110 min at room temperature the 260 kDa band from BBMV disappeared (Fig. 1(b)) but this band, as well as the new aminopeptidase N-complexed material was seen when BBMV were incubated with DMS (Fig. 1). Further incubation in DMS (to 17 h) culminated in the production of Coomassie-stained products in the stacking section of the gel with very little material entering the separating part of the gel. It is probable from these observations that DMS is acting to stabilise higher molecular weight complexes already present in BBMV but prone to degradation during storage. This seems very likely since the 260 kDa APN complex is already present in BBMV but increased in quantity in the presence of DMS. The production of the smallest (45 kDa) breakdown product of APN which still has the FD19 recognised isotope is also prevented on addition of DMS (Fig. 1, arrow 4), and perhaps also re-associated into higher molecular weight products as suggested from Fig. 1(c) where the 45 kDa product is no longer present.

The highest molecular weight complexes of APN are seen more clearly in FD19-immunoaffinity column purified DMS-treated BBMV. The scan is presented in Fig. 2. As well as the monomer, the 220 kDa and 260 kDa complexes were recognised. By comparison with the FD19 column purified APN from untreated BBMV (Fig. 5, scan a), it is apparent that complexes of the same M_r are normally present in BBMV but in smaller amounts. Thus DMS is increasing and stabilising associations between proteins which normally occur in BBMV.

Cross-linking of partially purified aminopeptidase N

APN is a glycoprotein and as such can be partially purified from solubilised BBMV using the lectin Concanavalin A (ConA). A time course of the cross-linking

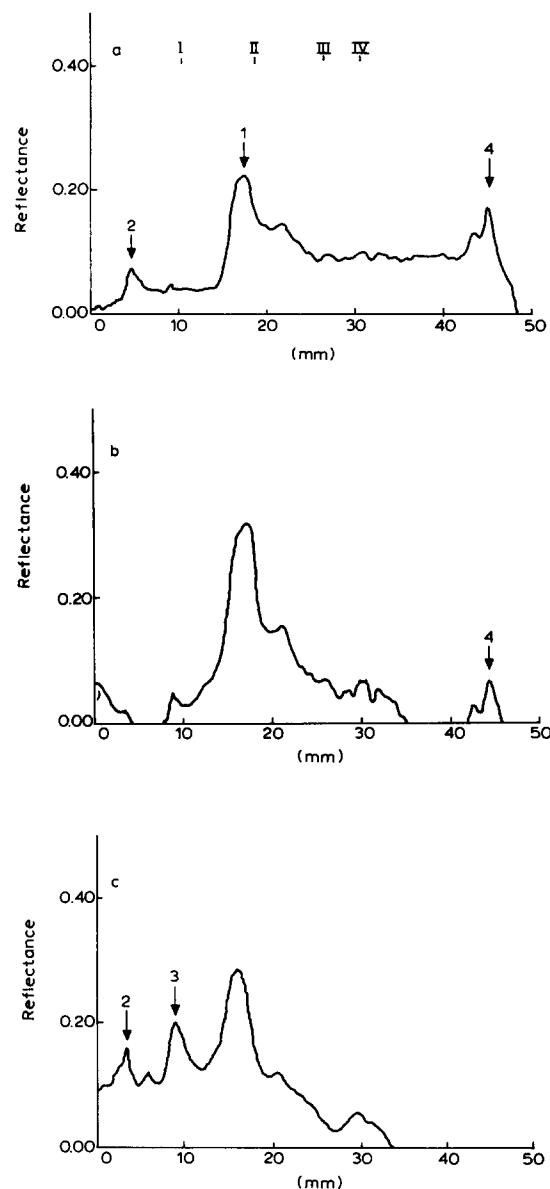


Fig. 1. Western blots using FD19 of BBMV in the presence and absence of DMS. Samples of BBMV (10 μ g protein) treated and untreated with DMS were removed at various time points, separated on SDS-PAGE (10%) and electroblotted onto nitrocellulose. These were blotted with FD19 and the Western blots scanned. Arrows 1 and 2 point to the 130 kDa monomer of APN and to the 260 kDa dimer. Arrow 3 points to a 220 kDa complex of APN. Scan a, BBMV at 0 min; scan b, BBMV at 110 min; scan c, BBMV plus DMS at 110 min. Molecular mass standards: I, 211 kDa; II, 119 kDa; III, 80 kDa; IV, 64 kDa.

of the ConA-purified fraction is presented in Fig. 3. Incubation of the ConA fraction with DMS resulted in the formation of the 220 and 260 kDa complexes within 5 min and these were stable even after 17 h. In BBMV (not shown), higher molecular weight material continued to be formed such that there was less material entering the separating part of the gels during the same time points. Thus the ConA glycoprotein fraction contains the components which associated with APN to

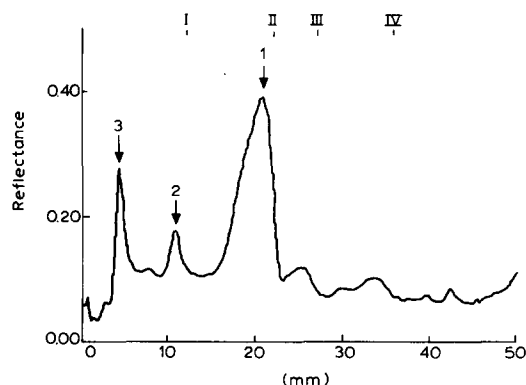


Fig. 2. Immunoaffinity (FD19) column purified products from DMS-treated BBMVs. BBMVs were treated with DMS (60 min, 20°C) and then solubilised and passed through an immunoaffinity column to remove aminopeptidase N and any aminopeptidase N complexes. A sample (8 µg) was run on SDS-PAGE (8%) and scanned. Arrow 1 points to APN monomer (130 kDa) and arrows 2 and 3 point to the 220 kDa and 260 kDa complexes, respectively. For a comparison with non cross-linked APN immunoaffinity column purified fraction, see Fig. 5(a). Molecular mass standards: I, 200 kDa; II, 116 kDa; III, 97 kDa; IV, 66 kDa.

form the 220 kDa, and 260 kDa complexes seen in BBMVs. That these were APN complexes was confirmed by blotting with FD19 (Fig. 4).

That the process of formation of high molecular weight complexes was not entirely random was confirmed by Western blot analysis of the 100 kDa protein seen in the ConA fraction and in BBMVs (data not shown). In BBMVs this band disappears within 30 min into a higher molecular weight complex as shown by blotting with the monoclonal antibody FD12 which specifically reacts with this polypeptide (described in Ref. 12). But in the ConA fraction the 100 kDa band was still visible after 17 h suggesting that the proteins it normally cross-links to in BBMVs are not present in the purified ConA fraction.

Cross-linking with DMS of the FD19 immunoaffinity column purified aminopeptidase N

Immunoaffinity column purified APN was treated with DMS and the proteins were separated by SDS-PAGE (Fig. 5). As can be seen from scan b, both the 220 kDa and 260 kDa complexes were made in quantity. This confirms that these complexes were composed of APN and any of its breakdown products. Therefore the 260 kDa complex seen in native BBMVs and increased by cross-linking is likely to be a dimeric form of APN. The 220 kDa complex may be APN cross-linked to one or more of its subunits.

Cross-linking of BBMVs with dimethyl 3,3'-dithiobis(propionimidate)

DTBP is a thiol-cleavable cross-linker. Cross-linking of BBMVs proteins and then immunopurifying APN complexes would allow analysis of any proteins cross-

linked to APN by subsequent thiol reduction of the cross-linker.

In preliminary experiments, it was found that under similar conditions DTBP cross-linked BBMVs proteins to a greater extent than did DMS. Fig. 6 shows scans of immunopurified APN complexes from DTBP cross-linked BBMVs. Without reduction of the cross-linker very little material entered the separating part of the gel suggesting that the APN was cross-linked to products of > 300 kDa (limit of size for entry into 8% gel). Since essentially only the 260 kDa and 45 kDa products of APN were released on reduction of the cross-linker, the bulk of APN appeared to be cross-linking to itself.

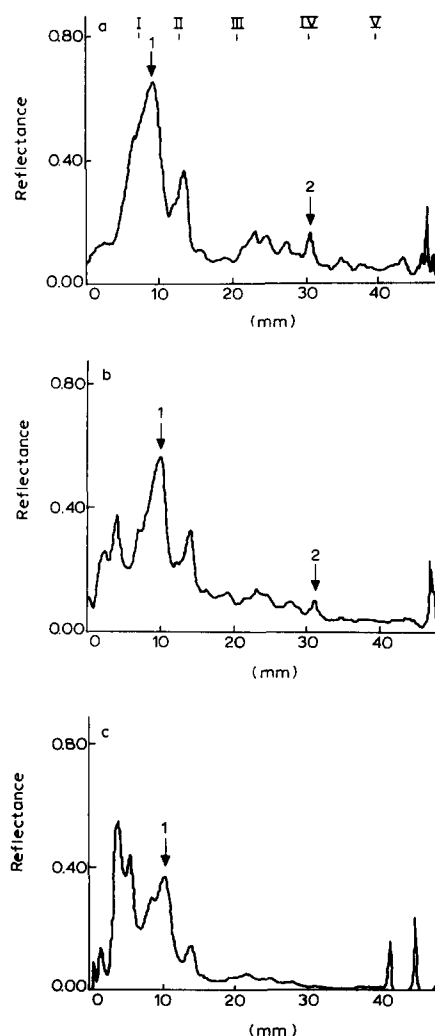


Fig. 3. Time course of cross-linking of concanavalin A partially purified aminopeptidase N. ConA column purified APN was incubated with DMS, aliquots (10 µg) were removed at various time points and analysed by SDS-PAGE (10%). The gels were scanned. Scans a, b and c represent ConA-purified fraction incubated for 0, 5 min and 17 h, respectively. The arrows point to APN monomer (1) and its 45 kDa breakdown product (2). Molecular masses are shown on scan a: I, 200 kDa; II, 116 kDa; III, 97 kDa; IV, 66 kDa and V, 45 kDa.

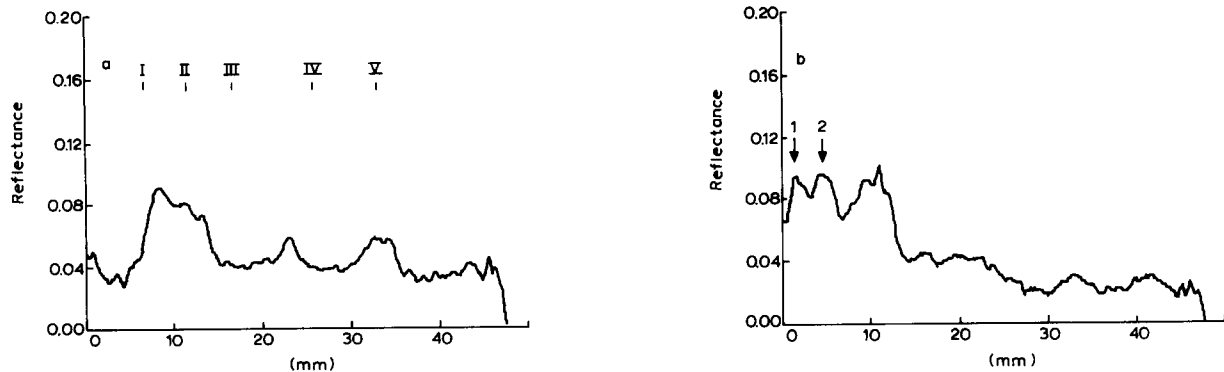


Fig. 4. Western blot (using FD19) of ConA fraction cross-linked with DMS. Samples were prepared as described in Fig. 3. Scans a and b represent ConA fraction treated with DMS for 0 and 30 min, respectively. Arrows point to cross-linked products of 220 kDa (1) and 260 kDa (2). The molecular mass markers are recorded on scan a and are: I, 211 kDa; II, 119 kDa; III, 98 kDa; IV, 60 kDa and V, 44 kDa.

It was of interest to determine whether APN could cross-link to any other membrane proteins *in situ*. Under conditions of limited proteolysis papain cleaves APN at the junctional peptide region thus removing a 125 kDa polypeptide containing the active site and the FD19 epitope into the supernatant (described in Ref. 12). The anchoring peptide region remains in the membrane. Papain digestion after cross-linking would remove the majority of the APN which is cross-linked to itself. Any APN cross-linked to other membrane peptides or cross-linked to substructures of itself which do not contain the papain site would remain in the membrane. In this way, effectively a concentration effect is achieved for the rarer cross-linking events.

A preliminary experiment using DMS showed by enzyme assay that more APN remained associated with membranes after cross-linking. Cross-linking decreased the activity of APN by 28% within 30 min. Normally, 10% of the enzyme activity remains associated with the membranes after papain treatment. Cross-linking followed by papain digestion increased the amount of APN remaining on the membranes by 130%.

BBMV treated with DTBP were subjected to limiting papain cleavage. The membrane pellets were solubilised and passed through a FD19 column. The FD19 recognised material was then analysed by SDS-PAGE and Western blots and the results are presented in Fig. 7. Comparing Fig. 7(a) with Fig. 6(b) shows that the number of bands between the dimer and monomer of APN has increased and a greater number of smaller polypeptides also appeared.

The released polypeptides from the above experiment were further analysed by passage through a FD19 column. Both the effluent and bound material were analysed on silver stained gels and the released polypeptides are shown in Fig. 8. Preparative gels were run and the 120 kDa, 95 kDa, 80 kDa, 66 kDa and 45 kDa polypeptides were subjected to N-terminal amino acid analysis. The 80 kDa polypeptide was N-terminally blocked and therefore no analysis is as yet available. The 120 kDa polypeptide had the same N-terminal sequence as rat dipeptidyl peptidase(IV) (Fig. 9(a)) [24]. The 66 kDa peptide had an N-terminal sequence which corresponded to human APN (Fig. 9(b)) from

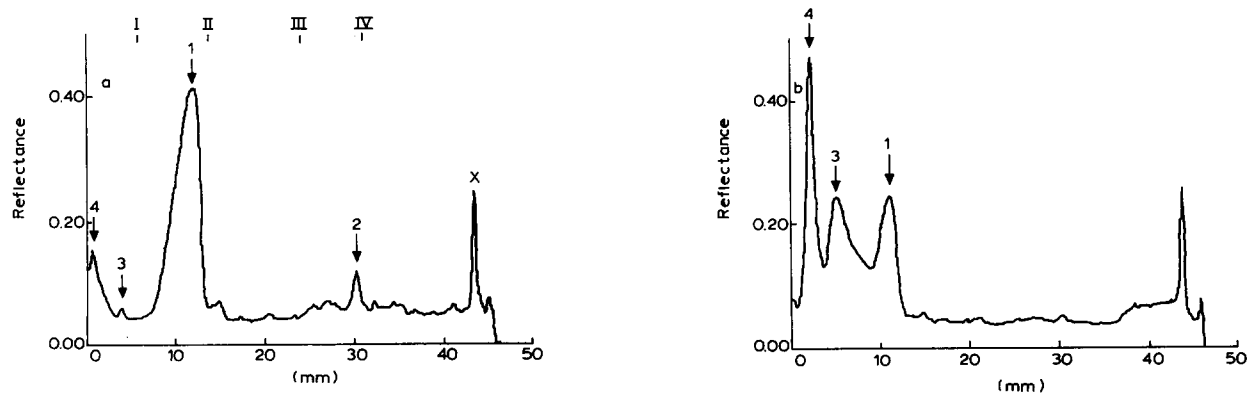


Fig. 5. Cross-linked with DMS of FD19 immunoaffinity column purified aminopeptidase N. An aliquot of column purified fraction was incubated with DMS (60 min, 22°C) and separated on SDS-PAGE (10%). Scan a is control FD19 purified APN (5 µg) and scan b is after cross-linked (5 µg protein). Arrows 1 and 2 point to APN monomer and its 45 kDa breakdown product, respectively. Arrows 3 and 4 point to the 220 kDa and 260 kDa complexes, respectively. Molecular mass markers: I, 211 kDa; II, 119 kDa; III, 64 kDa and IV, 44 kDa. X denotes the end of the gel.

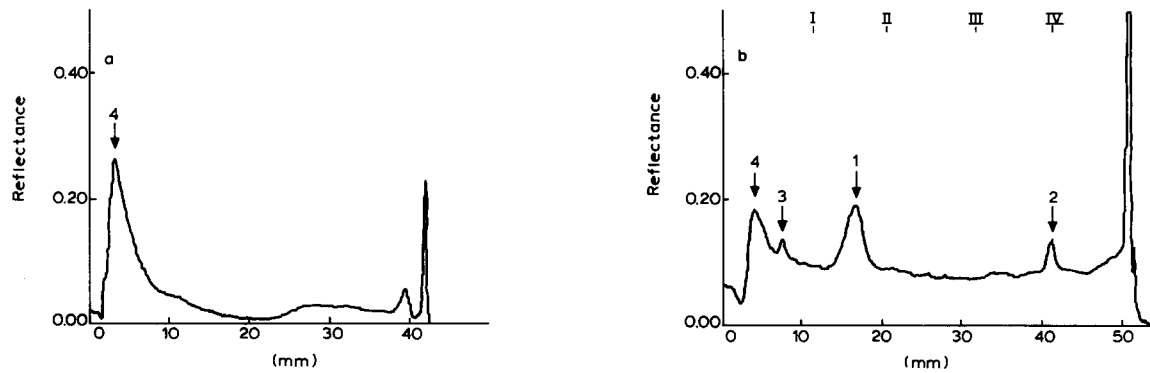


Fig. 6. Immunoaffinity column purified aminopeptidase N complexes from DTBP cross-linked BBMVs. DTBP-treated BBMVs were solubilised and passed through an FD19 column. Samples ($5 \mu\text{g}$) were resuspended in reducing or non-reducing buffer and subjected to SDS-PAGE (8%). Scan a, FD19 recognised protein from DTBP-treated BBMVs, non-reducing buffer b, and a, but treated in reducing buffer. Arrows point to APN monomer (1); 45 kDa breakdown product (2); 260 kDa dimer (3) and unreduced cross-linked material (4). Molecular mass standards: I, 200 kDa; II, 116 kDa; III, 66 kDa; IV, 45 kDa.

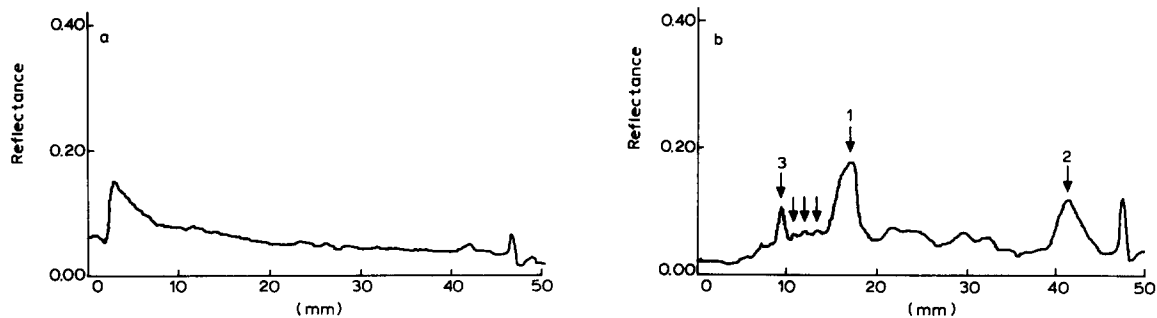


Fig. 7. Western blots using FD19, on papain cleaved DTBP-treated BBMVs. DTBP-treated BBMVs were digested with papain (10 min, 22°C), centrifuged, solubilised and passed through an FD19 column. Samples of FD19 recognised protein ($5 \mu\text{g}$) were resuspended in non-reducing or reducing buffer and electrophoresed through SDS-PAGE (8%) and Western blots using FD19 performed. Scan a, non-reduced APN complex; scan b, reduced APN complex. Arrows 1 and 2 point to APN monomer and its 45 kDa breakdown product, respectively. Arrow 3 points to the 260 kDa dimer and other arrows point to high molecular weight APN complexes.

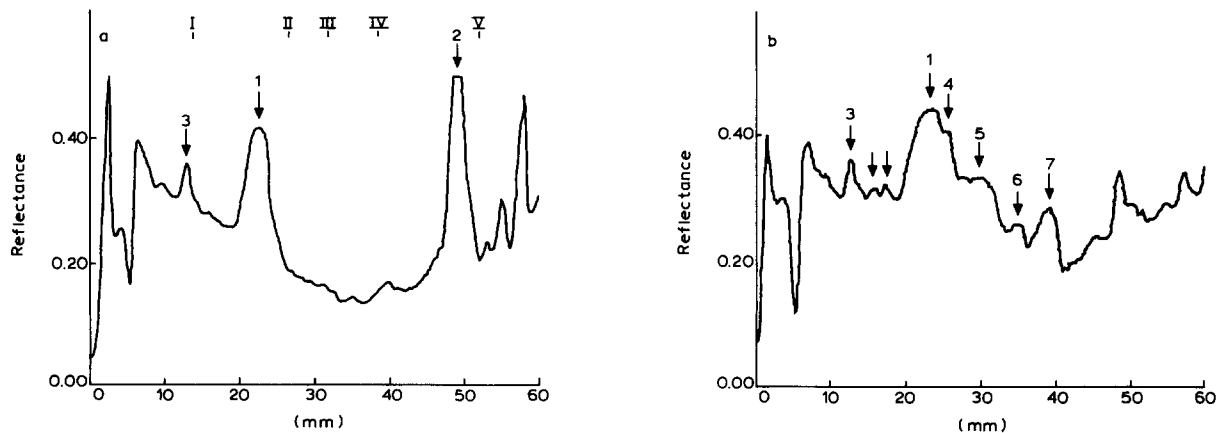


Fig. 8. Polypeptides from DTBP-formed aminopeptidase N complexes. BBMVs were cross-linked with DTBP and digested with papain. Proteins released from the membranes by papain were removed by centrifugation. Membrane pellets were solubilised and passed through an FD19 column. The eluted protein was dialysed, concentrated and resuspended in reducing buffer (15 min, $95-100^\circ\text{C}$). The reduced protein was again passed through an FD19 column and samples ($2 \mu\text{g}$) of the stuck (scan a) and effluent (scan b) material was electrophoresed through SDS-PAGE (8%). The gels were silver stained. Arrows point to APN monomer (1); 45 kDa breakdown product (2); 260 kDa dimer (3); 120 kDa polypeptide (4); 95 kDa polypeptide (5); 80 kDa polypeptide (6); and 66 kDa polypeptide (7). Molecular weight standards: I, 200 kDa; II, 116 kDa; III, 97 kDa; IV, 66 kDa; V, 45 kDa.

a.

Bovine: Met. Lys. Thr. Pro. Trp. Lys. Val. Leu. Ser. Gly. Leu. Leu. Arg.

Rat: Met. Lys. Thr. Pro. Trp. Lys. Val. Leu. Leu. Gly. Leu. Leu. Gly.

b.

Bovine: Thr. Pro. Ala. Asn. Glu. Val. Asn. Ile. Pro. Ala. Gln. Ile. Thr.

Human:.....Thr. Pro. Ala. Ser. Glu. Ile. Asn. Thr. Pro. Ala. Gln. Ile. Ser.

Bovine Glu. Met. Phe.....

Human Glu. Leu. Phe.....

c.

Bovine: Ser. Thr. Tyr. Leu. Leu. Ala. Tyr. Ile. Val.....

Human:Ser. Thr. Tyr. Leu. Leu. Ala. Phe. Ile. Val....

Fig. 9. N-terminal amino acid sequences of polypeptides. (a) 120 kDa polypeptide. The bovine renal 120 kDa polypeptide released upon reduction of FD19-purified DTBP-treated BBMV, aligns with the N-terminus of rat dipeptidyl peptidase IV precursor [24], with two differences (underlined). (b) 66 kDa polypeptide. The bovine renal 66 kDa polypeptide aligns with human intestinal APN [25] from amino acids 457 to 472, with five differences (underlined). (c) 45 kDa breakdown product and 95 kDa polypeptide. The bovine renal 45 kDa APN breakdown product and the 95 kDa polypeptide released upon reduction of FD19-purified DTBP-treated BBMV, align with the human APN [25] from amino acids 296 to 305 with one difference (Phe 203 for Tyr).

amino acids 457 to 472 [25]. Both the 95 kDa and the 45 kDa fragments had the same N-terminus (Fig. 9(c)), and this corresponded to the human APN sequence from amino acids 296 to 305 [25]. The 45 kDa fragment was sequenced from untreated BBMV.

Discussion

Bovine renal APN was cross-linked using two different bifunctional cross-linking reagents. The results show that the non-cleavable reagent DMS acted as a weaker cross-linker leaving a significant amount of the APN in the monomer state (130 kDa). The thiol-cleavable reagent DTBP cross-linked BBMV material to a greater extent and left no detectable APN monomer, dimer or other complexes of < 300 kDa. On cleavage of this cross-linker, the monomer and dimer were released. The span of the DMS molecule is 11 Å and that of DTBP is 11.9 Å. APN molecules therefore associate sufficiently closely in BBMV to allow extensive cross-linking.

Reduction of DTBP-APN complexes released a number of polypeptides in quantity and purity such that they could be subjected to automated N-terminal analysis. The 45 kDa product was derived from detergent-solubilised BBMV preparations. Since the N-terminal sequence corresponds to an internal sequence of human [25] and rat [26] APN it is a breakdown product of the 130 kDa APN monomer and may be formed by autolysis or cleavage by some other endogenous proteinase. This fragment was readily detected in Western blots and hence has the FD19-recognising epitope. The 95 kDa product is unlikely to have arisen from papain cleavage since it has the same N-terminus as the 45 kDa polypeptide. The peptide bond before this N-terminus is obviously labile and easily attacked. The 66 kDa product has a different sequence which is also found in the human APN and is hence also a breakdown product. This may have been produced by papain cleavage. Although the primary sequence of bovine renal APN has not yet been determined, the similarity of these internal sequences with those of

human intestinal APN [25] suggest that the sequence of this enzyme is highly conserved.

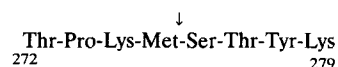
It is of interest that the 120 kDa released product proved to be dipeptidyl peptidase IV (DPP IV). Association between these two very similar ectoenzymes has not been previously reported. The products of DPP IV activity are dipeptides which can act as substrates for APN.

The above findings can be compared with previous work on intestinal APN. The 'subunits' seen with pig intestinal APN preparations have been characterised in terms of size on gels [8,10]. On the basis of peptide mapping of products released after cross-linking Svensson [10] postulated that the 168 kDa APN gave rise to 118 kDa and 54 kDa products and that the 54 kDa subunit was not a breakdown product of the 118 kDa species. The reported sizes of both the monomer and subunits are inconsistent with the present work on the bovine renal enzyme as well as other work [8].

Maroux et al. [8] reported the following sizes for pig intestinal APN: 130 kDa, 96 kDa and 48 kDa. This pattern of subunits was observed in trypsin or papain treated membranes or by spontaneous dissociation of APN. Other minor bands of 116 kDa, 76 kDa and 66 kDa bands were observed after proteolytic dissociation. It is possible that the 116 kDa band corresponded to the 120 kDa band now identified as dipeptidyl peptidase IV in the bovine renal system. The 96 kDa and 48 kDa bands could also correspond to the 95 kDa and 45 kDa products characterised in the present study.

A previous study on pig renal APN [11] suggested that aggregates of APN solubilised in Triton decompose into 280 kDa products which on further autolysis yield a product of 140 kDa. Trypsin dissociation of the aggregates yielded products of 140 kDa 95 kDa and 48 kDa.

From the above two studies, the 95 kDa and 45 kDa fragments may be common to both pig renal and intestinal forms of the enzyme. In bovine renal APN these two fragments have been shown to arise from cleavage of a specific common peptide bond. The cleavage site can be derived from the Fig. 9 and the known human APN sequence as between methionine and serine in the sequence:



This site is also conserved in the kidney APN sequence from rat [26].

In summary, bovine renal APN has been shown to form oligomeric structures and to associate with DPP IV. Apparent subunits dissociating from the oligomeric

structures have been shown to be breakdown products. The pattern of enzyme association and the formation of breakdown products may be similar to those reported in intestine.

Acknowledgement

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References

- 1 Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjöström, H., Noren, O. and Laude, H. (1992) *Nature* 357, 417-419.
- 2 Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellicchio, C.R., Shapiro, L.H., Look, T.A. and Holmes, K.V. (1992) *Nature* 357, 420-422.
- 3 Look, A.T., Ashmun, R.A., Shapiro, L.H. and Peiper, S.C. (1989) *J. Clin. Invest.* 83, 1299-1307.
- 4 Hussain, M.M., Tranum-Jensen, J., Noren, O., Sjöström, H. and Christiansen, K. (1981) *Biochem. J.* 199, 179-186.
- 5 Louvard, D., Maroux, S., Vannier, C. and Desnuelle, P. (1975) *Biochim. Biophys. Acta* 375, 236-248.
- 6 Kenny, A.J. and Maroux, S. (1982) *Physiol. Rev.* 62, 91-128.
- 7 Semenza, G. (1986) *Annu. Rev. Cell. Biol.* 2, 255-313.
- 8 Maroux, S., Louvard, D. and Baratti, J. (1973) *Biochim. Biophys. Acta* 321, 282-295.
- 9 Svensson, B., Sjöström, H. and Noren, O. (1982) *J. Biochem.* 126, 481-488.
- 10 Svensson, B. (1979) *Carlsberg Res. Commun.* 44, 417-430.
- 11 Wacker, H., Lehky, P., Vanderhaeghe, F. and Stein, E.A. (1976) *Biochim. Biophys. Acta* 429, 546-554.
- 12 Plakidou-Dymock, S., Tanner, M.J. and McGivan, J.D. (1993) *Biochem. J.*, in press.
- 13 Bertran, J., Werner, A., Moore, M.L., Stange, G., Markovich, D., Biber, J., Testar, X., Zorzano, A., Palacin, M. and Murer, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5601-5605.
- 14 Wells, R.G. and Hediger, M.A. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5596-5600.
- 15 Bertran, J., Maganin, S., Werner, A., Markovich, D., Biber, J., Testar, X., Zorzano, A., Kuhn, L.C., Palacin, M. and Murer, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5606-5610.
- 16 Biber, J., Steiger, B., Haase, W. and Murer, H. (1981) *Biochim. Biophys. Acta* 647, 169-176.
- 17 Lynch, A.M. and McGivan, J.D. (1987) *Biochim. Biophys. Acta* 899, 176-184.
- 18 Labbé, J.-P., Mornet, D., Roseau, G. and Kassals, R. (1982) *Biochem. J.* 21, 6897-6902.
- 19 Doyle, F.A. and McGivan, J.D. (1992) *Biochem. J.* 281, 95-102.
- 20 Hildreth, J.E.K. (1982) *Biochem. J.* 207, 363-366.
- 21 Laemmli, U.K. (1970) *Nature* 227, 680-681.
- 22 Towbin, H., Staehelin, T. and Gordon, J. (1976) *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.
- 23 Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.
- 24 Ogata, S., Misumi, Y. and Ikehara, Y. (1989) *J. Biol. Chem.* 264, 3596-3601.
- 25 Olsen, J., Cowell, G.M., Koenigshoefer, E., Danielsen, E.M., Moller, J., Laustsen, L., Hansen, O.C., Welinder, K.G., Engberg, J., Hunziker, W., Spiess, M., Sjöström, H. and Noren, O. (1988) *FEBS Lett.* 238, 307-314.
- 26 Watt, V.M. and Yip, C.C. (1989) *J. Biol. Chem.* 264, 5480-5487.