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Proteins of the Kidney Microvillar Membrane

THE AMPHIPATHIC FORM OF DIPEPTIDYL PEPTIDASE IV

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Dipeptidyl peptidase IV was solubilized from the microvillar membrane of pig kidney by Triton X-100. The purified enzyme was homogeneous on polyacrylamide-gel electrophoresis and ultracentrifugation, although immunoelectrophoresis indicated that aminopeptidase M was a minor contaminant. A comparison of the detergent-solubilized and proteinase (autolysis)-solubilized forms of the enzyme was undertaken to elucidate the structure and function of the hydrophobic domain that serves to anchor the protein to the membrane. No differences in catalytic properties, nor in sensitivity to inhibition by di-isopropyl phosphorofluoridate were found. On the other hand, several structural differences could be demonstrated. Both forms were about 130000 subunit mol.wt., but the detergent form appeared to be larger by no more than about 4000. Electron microscopy showed both forms to be dimers, and gel filtration revealed a difference in the dimeric mol.wt. of about 38000, mainly attributable to detergent molecules bound to the hydrophobic domain. Papain converted the detergent form into a hydrophilic form that could not be distinguished in properties from the autolysis form. A hydrophobic peptide of about 3500 mol.wt. was identified as a product of papain treatment. The detergent and proteinase forms differed in primary structure. Partial *N*-terminal amino acid sequences were shown to be different, and the pattern of release of amino acids from the *C*-terminus by carboxypeptidase Y was essentially similar. The results are consistent with a model in which the protein is anchored to the microvillar membrane by a small hydrophobic domain located within the *N*-terminal amino acid sequence of the polypeptide chain. The significance of these results in relation to biosynthesis of the enzyme and assembly in the membrane is discussed.

Dipeptidyl peptidase IV (EC 3.4.14.– or 3.4.21.–) is one of eight peptidases located in the kidney or intestinal microvillar membrane (for review, see Kenny & Booth, 1978). The enzyme is an intrinsic membrane protein requiring the use of a proteinase (e.g. papain), autolysis at pH 3.8, or detergents to solubilize it from the membrane. Previous studies on the purified kidney enzyme (Hopsu-Havu *et al.*, 1968; Barth *et al.*, 1974; Kenny *et al.*, 1976; Yoshimoto & Walter, 1977) have been made with the form solubilized by autolysis. The enzyme when so purified is a serine peptidase very sensitive to inhibition by di-isopropyl phosphorofluoridate. It is dimeric, comprising two identical subunits of about 130000 mol.wt., each containing a reactive serine at the catalytic site (Kenny *et al.*, 1976). However, when purified in this way, part of the native protein, a hydrophobic domain, has been lost during solubilization and remains attached to the membrane. A form solubilized by non-ionic detergents might be expected to resemble the native enzyme, except for the substitution of detergent molecules for the membrane lipids formerly contiguous with the hydrophobic domain that anchored the enzyme to the membrane. A comparison of the properties of two preparations of the enzyme, the autolysis and the detergent forms, can give some insight into the structure and function of the hydrophobic domain. The present paper describes the purification of the detergent form of dipeptidyl peptidase IV after solubilization with Triton X-100. The results indicate that the hydrophobic domain is located within the first 30–40 residues from the *N*-terminus of the polypeptide chain. Some of this work has been presented in preliminary form (Kenny *et al.*, 1977; Kenny *et al.*, 1978).

Materials

The source of pig kidneys and other materials, except where otherwise noted, were as previously described (Kenny *et al.*, 1976). The following were obtained from the sources indicated. Triton X-100 (scintillation grade), Brij 35, sodium deoxycholate,

cetyltrimethylammonium bromide, polyamide layer sheets from BDH Chemicals Ltd., Poole, Dorset, U.K.; Tween 20 from Sigma (London) Chemical Co. Poole, Dorset BH17 7NH, U.K.; Lubrol WX from ICI Ltd., Piccadilly Plaza, Manchester, U.K.; Bolton & Hunter (1973) reagent (code IM 86), *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate from The Radiochemical Centre, Amersham, Bucks., U.K.; carboxypeptidase Y (from baker's yeast) from Pierce & Warriner (U.K.) Ltd., Chester CH1 4EF, Cheshire, U.K.; Fluorescamine (Fluram Roche) from Roche Products Ltd., London W1M 6AP, U.K.; phenyl-Sepharose CL-4B from Pharmacia (G.B.) Ltd., London W.5, U.K.; Guanidinium chloride (BDH) was recrystallized as described by Nozaki & Tanford (1967).

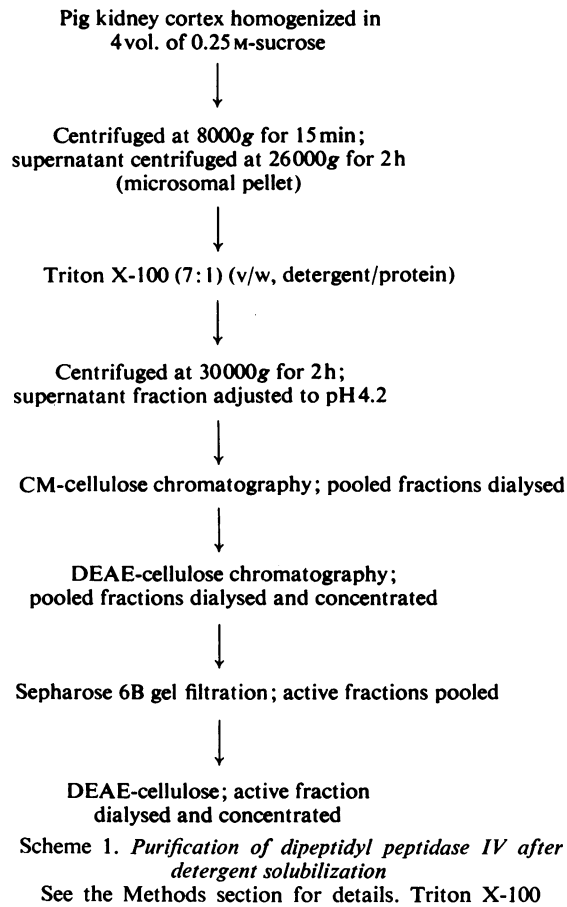
Methods

Trials of detergents

A microsomal fraction was prepared (see below) and resuspended in 5mM-Tris/HCl buffer, pH7.0, to a concentration of 5.4mg of protein/ml. Stock solutions of detergents were added to 0.5ml samples of the suspension to give a range of concentrations, 0.05–2.0% when the volume was adjusted to 2.0ml. The mixtures were shaken for 1h at 20°C and centrifuged for 1h at 100000*g*. The supernatant fractions were assayed for protein and various microvillar enzyme activities. Samples (0.1ml) of the supernatant fractions were loaded on to 7.7% (w/v) polyacrylamide gels (Davis, 1964), and electrophoresed until the dye (Bromophenol Blue) front had nearly reached the bottom of the tube. The gels were removed, sliced into 10mm pieces, added to tubes containing 1.5ml of 5mM-Tris/HCl, pH7.0, and left for 20h at 20°C before assaying for enzyme activity.

Purification of the detergent (Triton X-100) form of dipeptidyl peptidase IV from pig kidney

The method is summarized in Scheme 1. Cortical tissue (2kg) was homogenized with a Kenwood blender (model A956A) in batches in 0.25M-sucrose for 2min to give a 20% (w/v) homogenate. After removing the pellets sedimenting at 8000*g* for 15min, the supernatant fraction was centrifuged at 26000*g* for 2h. This microsomal pellet was resuspended in about 500ml of 5mM-Tris/HCl, pH7.9, and Triton X-100 added to give a final volume of 800ml and a detergent/protein (v/w) ratio of 7:1. The suspension was stirred for 1h at 20°C and centrifuged at 30000*g* for 2h. (The concentrations of Triton X-100 are given throughout as v/v.) The supernatant was brought to pH4.2 by the addition of 2vol. of 10mM-sodium acetate/acetic acid buffer, pH4.2, and adjusted, if necessary, with 8.7M-acetic acid. About 500g of CM-cellulose (CM-52) was equilibrated with the same buffer, packed into a column (diameter 85mm)



Scheme 1. *Purification of dipeptidyl peptidase IV after detergent solubilization*

See the Methods section for details. Triton X-100 (0.1%–0.15%) was present in buffers used at each chromatographic step.

and the supernatant pumped on to the column (150ml/h). After loading, it was washed with 2 litres of the same buffer now containing 0.15% Triton X-100. A linear gradient derived from 1000ml of the wash buffer and 1000ml of 200mM-sodium acetate/acetic acid buffer, pH5.8, 0.15% Triton X-100 was used for elution. The active fractions were pooled and dialysed for 20h against 2 × 5 litres of 5mM-Tris/HCl, pH7.9, 0.15% Triton X-100, and pumped on to a column (25mm × 300mm) of DEAE-cellulose DE-52 previously equilibrated with the same Tris buffer. The column was eluted by a linear gradient (vol. 1.5 litres) of NaCl (0–200mM) in the same buffer containing 0.15% Triton X-100. The pooled fractions containing the activity were dialysed against 2 × 5 litres of 5mM-Tris/HCl buffer, pH7.9, 0.1% Triton X-100 and concentrated by loading on to a small (9mm × 50mm) column of DEAE-cellulose, equilibrated as before, and eluted in about 6ml with

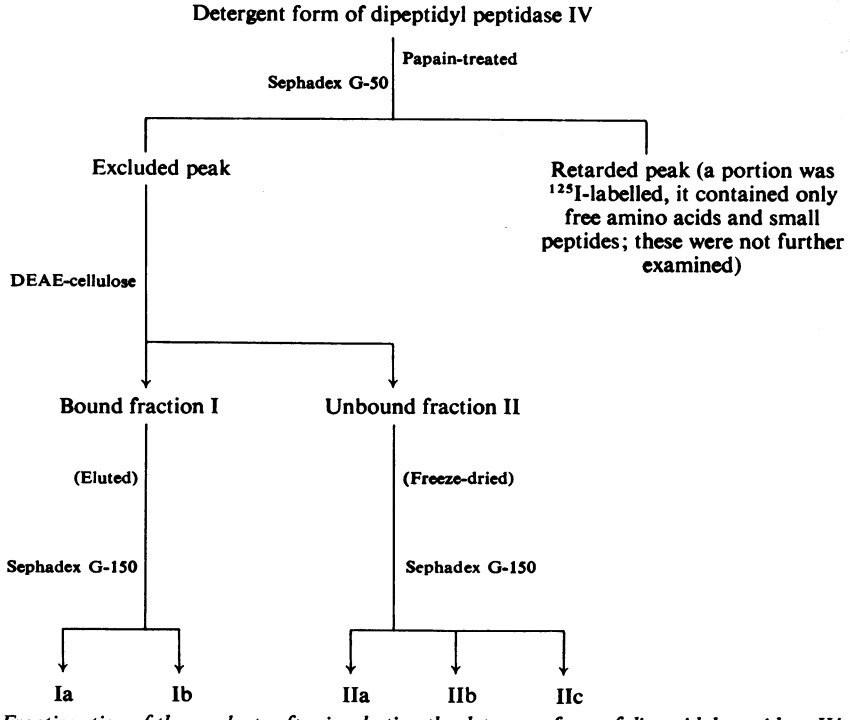
300mM-NaCl in 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100. The concentrated sample was then applied to a column of Sepharose 6B (50mm × 900mm) equilibrated and developed with 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100. The active fractions were pooled and pumped on to a column (9mm × 50mm) of DEAE-cellulose and eluted by 400ml of a linear gradient (0–100mM) of NaCl in the same buffer. The active fractions were pooled, dialysed against 5 litres of 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100 and concentrated as before to a volume of about 5ml. All the purification steps were carried out at 5°C. The enzyme was stored at −70°C. Some batches were dialysed against 5 litres of 0.1% Triton X-100 to remove salts. A Sorval RC-5 centrifuge was used with GS3, GSA and SS34 rotors.

Papain digestion of dipeptidyl peptidase IV

Papain was activated by incubating, at 37°C for 15min, 0.2ml of 25mM-2-mercaptoethanol, 0.1ml of papain (25mg/ml) with 0.7ml of 0.1M-Pipes (1,4-piperazinediethanesulphonic acid) adjusted to pH6.8 with NaOH. Samples of activated papain were added to samples of the autolysis or detergent-solubilized forms of the enzyme (50μg of papain/mg of enzyme) and incubated at 37°C for 1.5h. The reaction was stopped by the addition of a slight molar excess (in relation to thiol) of solid iodoacetamide. When necessary, the inactive papain was removed from the mixture by chromatography on Sephadex G-150.

Fractionation of the products in the incubation mixture after treatment of the detergent form of dipeptidyl peptidase IV with papain

The methods are summarized in Scheme 2. The autolysis form of the enzyme was used as a control. A sample (8mg of protein, 62nmol, monomeric mol.wt. ,130000) of the detergent form was incubated with papain as described above. The mixture was applied to a column of Sephadex G-50 (25mm × 400mm) equilibrated with 5mM-*N*-ethylmorpholine/acetic acid buffer, pH8.0, 0.1% Triton X-100 and developed with the same medium. The fractions (3.5ml) were monitored for dipeptidyl peptidase activity and for protein and peptides with fluorescamine. The retarded fluorescamine-positive material (eluting at about 0.85 of the column volume) was in part labelled with ^{125}I and examined by high-voltage paper electrophoresis (see below). The excluded fraction (0.35 column volume) was loaded on to a



Scheme 2. *Fractionation of the products after incubating the detergent form of dipeptidyl peptidase IV with papain*
See the Methods section for details. All buffers contained 0.1% Triton X-100. Fractions Ib, IIa and IIc were labelled with ^{125}I by using the Bolton & Hunter (1973) reagent.

column of DEAE-cellulose (9mm × 50mm) equilibrated with the same *N*-ethylmorpholine medium and, after washing with 10ml of the medium, was eluted with 4ml of the 0.2M-*N*-ethylmorpholine/acetic acid buffer (pH8.0)/0.1% Triton X-100, to give fraction I. The unbound fluorescamine-positive material in the washings, designated fraction II, was concentrated by freeze-drying. Each of the two fractions was applied to a column of Sephadex G-150 (25mm × 960mm) equilibrated and developed with 5mM-*N*-ethylmorpholine/acetic acid buffer (pH8.0)/0.1% Triton X-100. The eluate (5ml fractions) was monitored as before. Fraction I resolved into fractions [Ia (0.41 column volume) and Ib (0.97 column volume)], and fraction II into three fractions [IIa (0.38 column volume), IIb (0.80 column volume) and IIc (0.97 column volume)]. Fractions Ib, IIa and IIc were freeze-dried and each redissolved in 4ml of water and labelled with ^{125}I as described below.

Labelling of proteins and peptides with ^{125}I

Samples (15mg) of α-lactalbumin and insulin B-chain were iodinated with Na^{125}I by using chloramine-T, by a method based on that used by Greenwood *et al.* (1963); 0.5mCi of Na^{125}I was used with carrier in the ratio 8μmol of NaI/15mg of polypeptide. After labelling, the sample of α-lactalbumin was extensively dialysed against water; insulin B-chain was applied to a column (9mm × 600mm) of Sephadex G-50, eluted with 0.1% NaI, and the excluded peak of radioactivity collected.

Dipeptidyl peptidase IV and the peptide fractions from the treatment with papain were labelled with the Bolton & Hunter (1973) reagent. Samples of the reagent containing about 30μCi were placed in tubes and the solvent (benzene) removed by a stream of N_2. Samples (1mg) of the detergent and proteinase forms of the enzyme were freeze-dried and resuspended in 20μl of 0.1M-H_3BO_3/NaOH buffer, pH8.5, and added to the dried reagent. The mixture was agitated at 0°C for 15min. Glycine (0.5ml of an 0.2M solution in borate buffer) was added and agitated for 5min at 0°C. The mixture was loaded on to a column (25mm × 400mm) of Sephadex G-50 equilibrated with 5mM-*N*-ethylmorpholine/acetic acid buffer, pH8.0, and developed with the same buffer. Peptides were treated in the same manner, except that column buffer contained 0.1% Triton X-100. The radioactivity in the excluded fractions was pooled and freeze-dried.

Determination of end groups and partial sequence of the detergent and autolysis forms of dipeptidyl peptidase IV

Samples of the enzyme (13mg, 100nmol, monomeric mol.wt.) in 2ml of 5mM-Tris/HCl, pH7.9, were brought to a concentration of 8M with guanidinium chloride and 50mM with dithiothreitol. The final volume was 10ml. In the sample subjected to the manual subtractive Edman degradation, recrystallized urea was substituted for guanidinium chloride. The samples were sealed and left for 1h at 20°C. Iodoacetic acid was added to give a final concentration of 120mM. The mixture was dialysed exhaustively against water and freeze-dried. *N*-Terminal residues were identified by dansylation in 8M-urea of 1mg samples of the reduced carboxymethylated protein as described by Gros & Labouesse (1969). After hydrolysis the dansyl derivatives were identified on 50mm × 50mm polyamide layer sheets as described by Hartley (1970).

C-Terminal amino acids were investigated by digestion of 5mg of the denatured reduced carboxymethylated protein with 50μg of carboxypeptidase Y in 100mM-pyridine/acetic acid buffer, pH5.0. The incubation volume was 2.5ml. Samples (0.5ml) were removed after 0, 15, 90 and 180 min incubation at 37°C, treated with 0.5ml of 20% (w/v) trichloroacetic acid, centrifuged to remove protein and the supernatants extracted with 10ml of diethyl ether to remove trichloroacetic acid. The aqueous residues were freeze-dried and analysed for amino acids.

The sequence of the *N*-terminal region of the autolysis form was determined in a 'spinning-cup' automatic sequencer (Beckman 890C). Duplicate samples (10mg) of the reduced carboxymethylated form of the autolysis-solubilized enzyme were subjected to 16 degradative steps. The methods were those described by Gigli *et al.* (1977). The detergent-solubilized form was sequenced in duplicate samples (9mg) by the manual subtractive Edman degradation coupled to dansylation as described by Gray (1967).

Amino acid analysis

These were performed by using a Rank–Hilger Chromospek analyser.

Carbohydrate analyses

Samples (1mg) of the detergent and proteinase forms (the latter after papain treatment of the detergent form) were dialysed exhaustively against distilled water and analysed by g.l.c. as described by Bhatti *et al.* (1970) and Clamp *et al.* (1971).

Protein determinations

Protein was determined by the Lowry method as previously described (Kenny *et al.*, 1976). Interference appearing in samples containing Triton X-100, Lubrol WX, Brij 35 and Tween 20 was prevented by adding sodium dodecyl sulphate to the sample [to 2% (w/v) concentration] before assay.

The column fractions from the analysis of the products of papain treatment of dipeptidyl peptidase IV were monitored for peptides and protein by the fluorescamine reaction, performed as described by Udenfried *et al.* (1972). Samples of the column

fractions were also hydrolysed before assay in order to amplify the fluorescence; 0.5ml of 10M-NaOH was added to the sample and heated in a boiling-water bath for 2h. After cooling, the contents were neutralized by the addition of 12M-HCl and assayed as before. Fluorescence was determined in an Aminco–Bowman SPF 125 spectrophotofluorimeter and results expressed in arbitrary units.

Chromatography on phenyl-Sepharose CL-4B

Small columns (4mm × 20mm) containing about 0.5ml of phenyl-Sepharose CL-4B were washed with 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100. Samples (0.2ml) of detergent and proteinase-solubilized enzymes (10μg of protein) were added and the column was eluted first with the same buffer and then with buffer containing 50% (v/v) ethylene glycol, at a rate of about 0.5ml/min; 0.1ml fractions were collected and assayed for enzyme activity. Some enzyme samples were ^{125}I-labelled by the Bolton & Hunter (1973) reagent, the eluates then being monitored for radioactivity. Peptide fractions derived from papain treatment of the detergent form of dipeptidylpeptidase IV were analysed in the same way. Each had been ^{125}I-labelled and the samples contained about 10000 c.p.m. of radioactivity.

Extraction of peptides with chloroform/methanol

The method was based on that of Bligh & Dyer (1959). Samples of peptides Ib, IIa and IIc (0.1ml containing about 10000 c.p.m. of ^{125}I radioactivity) were diluted to 1ml with water and mixed with 1.25ml of chloroform and 2.5ml of methanol, and shaken for 5min. A further 1.25ml of chloroform was added and shaken again for 5min and 2.5ml of water added and reshaken. After brief gentle centrifugation, a sample of the upper aqueous layer was removed. The organic phase below the interface was also removed. Both phases were assayed for radioactivity. The results were expressed as the fraction (%) of the added radioactivity recovered in the organic phase.

Other methods

The following methods were those described previously (Kenny *et al.*, 1976): polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate; assay of dipeptidyl dipeptidase IV with glycyl-L-proline 2-naphthylamide and L-alanyl-L-alanine 2-naphthylamide; purification of the autolysis solubilized form of dipeptidyl peptidase IV; labelling of the enzyme with di-isopropyl [^{32}P]phosphorofluoridate; molecular-weight determinations on columns of Sepharose 6B; sedimentation-velocity determinations in the ultracentrifuge; electron microscopy; determination of protein; assay of radioactivity; preparation of buffers; high-voltage paper electrophoresis.

Polyacrylamide-gel electrophoresis in the presence of organic base dodecyl sulphate was carried out as described by Booth (1977).

Immunological methods, including the preparation of immunoglobulins, crossed immunoelectrophoresis and crossed charge-shift immunoelectrophoresis are described in the following paper (Booth *et al.*, 1979).

Results

Solubilization of microvillar membrane enzymes by detergents

The objectives in solubilizing microvillar membrane enzymes were to release them without loss of activity and in a form in which they could be purified by conventional means. In this respect we observed, at an early stage, that failure of the activity to sediment when centrifuged at 100000*g* for 60min was not necessarily a good criterion for these objectives. The demonstration that the activities could, after detergent treatment, penetrate a 7.7% polyacrylamide gel during electrophoresis (Davis, 1964) was a more reliable criterion. Some of the detergents examined proved unsatisfactory. Sodium dodecyl sulphate and cetyltrimethylammonium bromide inactivated the peptidases at a concentration of 0.3% (w/v). Although Brij 35, Tween 20 and deoxycholate released the peptidases, without loss of activity, in a non-sedimentable form, significant amounts of the activities did not penetrate the 7.7% polyacrylamide gels during electrophoresis. Only Lubrol WX and Triton X-100, of the detergents tested, were effective in solubilizing peptidases according to this criterion. These two detergents at a concentration of 0.75%, corresponding to a detergent/microsomal protein ratio of 6:1, were found to solubilize aminopeptidase M (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), γ-glutamyltransferase (EC 2.3.2.2), dipeptidyl peptidase IV (EC 3.4.14.– or 3.4.21.–) and neutral endopeptidase (EC 3.4.24.–) in satisfactory amounts. The results varied for different enzymes, but generally 25–90% of the applied activity penetrated the 7.7% gels.

Purification of the detergent form of dipeptidyl peptidase IV

The steps were similar to those used for the purification of the autolysis form of the enzyme (Kenny *et al.*, 1976) and are described in the Methods section (Scheme 1). Triton X-100 was added to a resuspended microsomal pellet in a detergent/protein ratio of 7:1. The soluble material was separated from the residual pellet by centrifugation, and fractionated on columns of CM-cellulose, DEAE-cellulose, Sepharose 6B and a final one of DEAE-cellulose. Triton X-100 (0.1–0.15%) was present in the buffers at each step. So far, ten batches of the enzyme have been purified in this way; the results for one preparation are shown in Table 1. The detergent form of the

Table 1. *Purification of the detergent form of dipeptidyl peptidase IV*
See the Methods section for details of each step. One unit of peptidase activity is defined as 1 μmol of 2-naphthylamine released from the substrate (glycyl-L-proline 2-naphthylamide)/min.

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg of protein)	Purification (fold)
Homogenate	6450	221000	7140	100	0.0322	1
Microsomal fraction	550	26200	3540	49.5	0.135	4
Triton X-100 supernatant	880	15500	2820	39.4	0.182	6
CM-cellulose	750	734	1320	18.4	1.79	56
DEAE-cellulose (1)	167	146	788	11	5.39	167
Sepharose 6B	217	85	759	10.6	8.93	277
DEAE-cellulose (2)	127	57	698	9.8	12.2	380
DEAE-cellulose (concentrated enzyme)	7.2	46.6	649	9.1	13.9	432

enzyme behaved in a similar manner to the autolysed form during these fractionation steps, although on DEAE-cellulose the activity eluted at a slightly higher point on the NaCl gradient than did the autolysis form. Rather unexpectedly, we observed that Triton X-100 could be omitted from the buffers in the steps after Sepharose 6B, without detriment to the preparation. The presence of detergent was essential at earlier stages; without it, aggregation occurred, and little or no activity could be eluted from the columns. Each batch of enzyme was characterized by polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate. They were found to be single-banded when stained with Coomassie Brilliant Blue, even when 20 μg of protein was applied to each track (Plate 1*a*). Crossed-immunoelectrophoresis, in which the proteins were electrophoresed in the second dimension into agarose containing rabbit immunoglobulins raised to detergent-solubilized pig microvillar membrane proteins, revealed the presence of a minor contaminant, which, by specific staining, was shown to be aminopeptidase M. This is the principal antigen in the microvillar membrane and consequently the immunological method is very sensitive for the detection of aminopeptidase M.

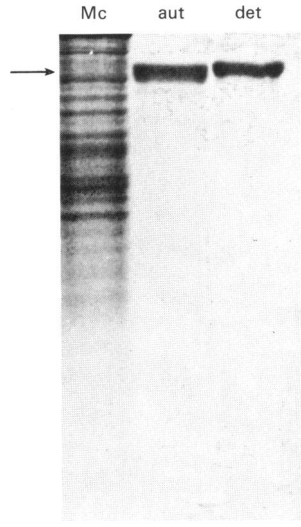
The autolysed form was reported to have endopeptidase activity by virtue of its ability to hydrolyse substrates, such as Z(= benzyloxycarbonyl)-Gly-Pro-Leu-Gly-Pro (Kenny *et al.*, 1976). The demonstration of this type of specificity required very high enzyme-to-substrate ratios and incubation times of 4h or more. The site of cleavage of the peptides involved the carboxyl function of proline residues and the hydrolysis was abolished by di-isopropyl phosphorofluoridate. For these reasons the endopeptidase activity was attributed to dipeptidyl peptidase IV, since at that time the properties of another enzyme, present in the kidney cytosol, with this specificity, 'post-proline-cleaving enzyme', did not indicate that it was a serine peptidase (Walter, 1976). Later the same laboratory reported that the cytosol endopeptidase was sensitive to di-isopropyl phosphorofluoridate (Walter & Yoshimoto, 1977). The endopeptidase activity in our preparations of dipeptidyl peptidase IV has been recently re-investigated (J. Ingram & A. J. Kenny, unpublished work) with the finding that the two activities can be differentially affected by both heat treatment and di-isopropyl phosphorofluoridate (the dipeptidyl peptidase activity being the more sensitive in each case). It is clear that the endopeptidase activity is attributable to a minor contamination by another enzyme, most probably the 'post-proline-cleaving enzyme'. Assays of the two activities suggested that the ratio of the endopeptidase activity to dipeptidyl peptidase activity in our preparation was 0.0006 : 1.

Comparison of the catalytic properties of the detergent and autolysis form of dipeptidyl peptidase IV

The values of K_m and $V_{max.}$ for the two enzyme forms are given in Table 2 and show no significant differences. The titration curves obtained with di-isopropyl phosphorofluoridate were also essentially the same for each form. Moreover, the slightly increased sensitivity to the inhibitor observed with the autolysis form when L-alanyl-L-alanine 2-naphthylamide, rather than glycyl-L-proline 2-naphthylamide, was substrate (Barth *et al.*, 1974; Kenny *et al.*, 1976) was also seen with the detergent form.

Comparison of the structural properties of the detergent and proteinase forms of dipeptidyl peptidase IV

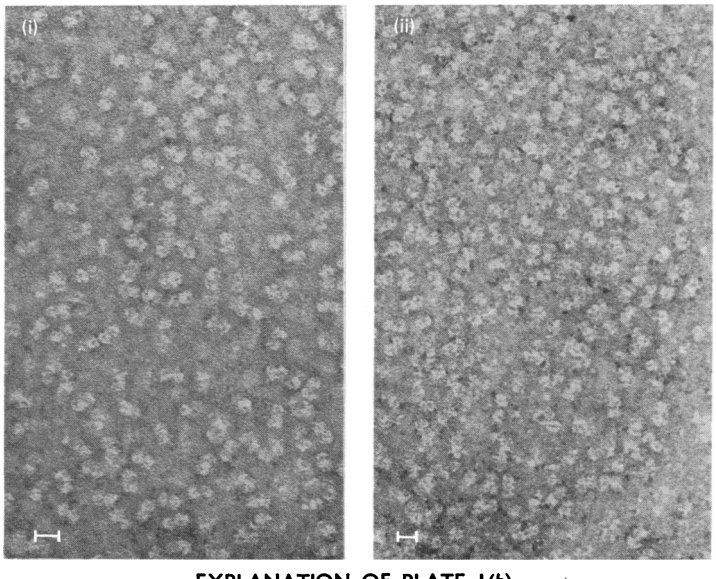
The proteinase form described in this section has been generated by either of the two methods, autolysis or treatment with papain. The autolysis form had been purified after an autolytic step early in the preparation as previously described (Kenny *et al.*, 1976). The papain form was obtained by limited proteolysis



EXPLANATION OF PLATE 1(*a*)

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

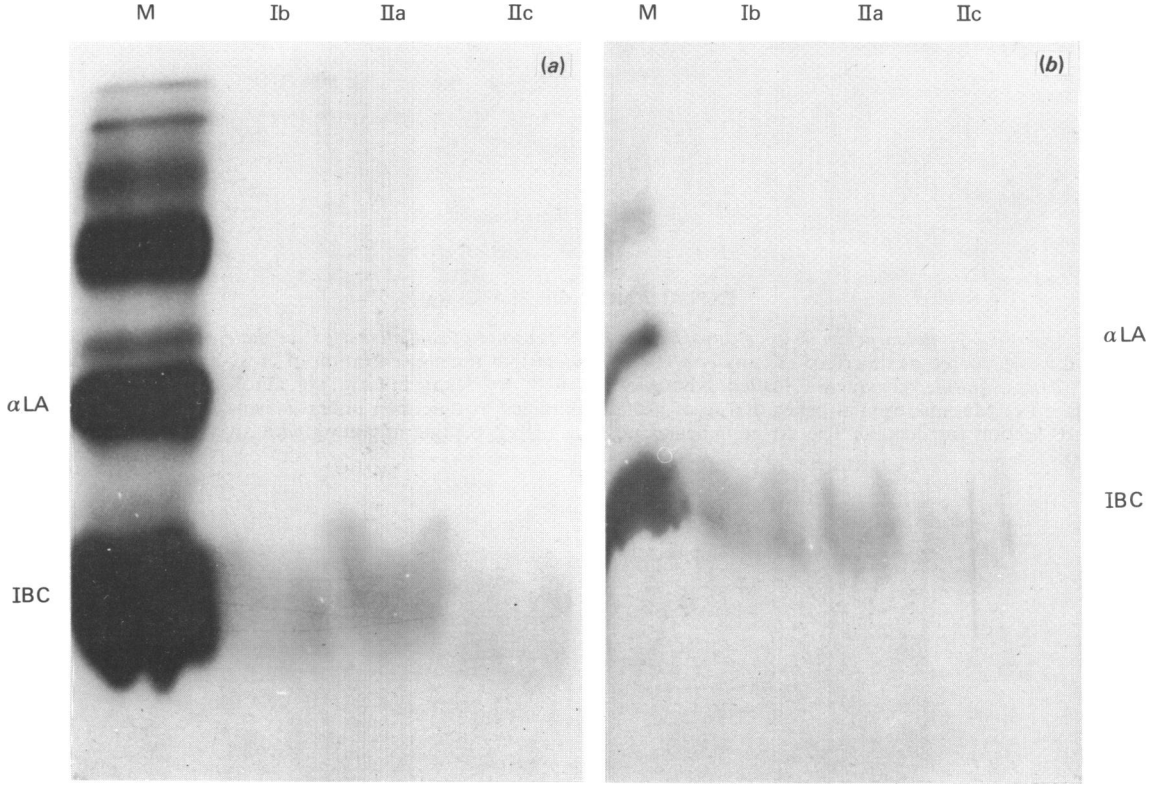
The gel was prepared as described (Kenny *et al.*, 1976) except that the concentration of cross-linker was increased 2-fold; 20μg of purified enzyme was loaded; Abbreviations used: det, detergent form; aut, autolysis form of dipeptidyl peptidase IV; Mc, microsomal pellet, 100μg of protein, obtained as described under enzyme purification (see the Methods section for details). The arrow indicates position of polypeptide migrating with an apparent mol.wt. of 130000.



EXPLANATION OF PLATE 1(*b*)

Electron micrographs of the autolysis (*i*) *and proteinase* (*ii*) *forms of dipeptidyl peptidase IV*

The preparations were negatively stained with uranyl acetate (Kenny *et al.*, 1976). The horizontal bar represents 20nm. Both forms of the enzyme appear as dimers, each subunit being about 7nm in diameter and often appearing to be hollow or dimpled.



EXPLANATION OF PLATE 2

Radioautographs of peptidase Ib, IIa and IIc after electrophoresis in the presence of dodecyl sulphate

The slab gels were prepared with 20% (w/v) acrylamide and the electrophoretic system was that described by Booth (1977). (*a*) Gel stained for protein washed, dried and exposed for 21 days; (*b*) gel dried without staining or washing and exposed for 36h. The iodinated markers (tracks M) were insulin B-chain (IBC; 3500 mol.wt.) and α-lactalbumin (αLA; 14500 mol.wt.). The long exposure in (*a*) has revealed some heterogeneity in the iodinated α-lactalbumin.

Table 2. *Comparison of the kinetic constants (K_m and $V_{max.}$) for the detergent and autolysis forms of dipeptidyl peptidase IV*
$V_{max.}$ is expressed as μmol of substrate hydrolysed/min/mg of protein, and K_m as μM; each ± S.D. The number of experiments is shown in parentheses. The colorimetric assay for 2-naphthylamine was used (Kenny *et al.*, 1976).

Substrate ...	Gly-Pro-2-naphthylamide		Ala-Ala-2-naphthylamide	
Enzyme form	$V_{max.}$	K_m	$V_{max.}$	K_m
Detergent	52 ± 4.2(5)	493 ± 48(5)	2.88 ± 0.32(4)	1057 ± 120(4)
Autolysis	59 ± 7.0(6)	578 ± 119(6)	2.74 ± 0.60(4)	977 ± 78(4)

Table 3. *Determination of molecular weight and Stokes radius by gel filtration on a column of Sepharose 6B*
The column dimensions were 50mm × 900mm; the column was calibrated and run as described previously (Kenny *et al.*, 1976). The buffer was 5mM-Tris/HCl, pH 7.9, containing, in some experiments, Triton X-100. Stokes radius was calculated by the correlation of Laurent & Killander (Siegel & Monty, 1966). Abbreviations used: DPP-det, DPP-aut, DPP-det-pap, detergent, autolysis and papain-treated detergent forms respectively of dipeptidyl peptidase IV. Results are means (± S.D.), with the number of determinations in parentheses. Four batches of DPP-det and one batch of DPP-aut were studied.

Expt.	Triton X-100 in buffer (%)	10^{-3} × Mol.wt.			Stokes radius (nm)		
		DPP-det	DPP-aut	DPP-det-pap	DPP-det	DPP-aut	DPP-det-pap
1	0	324	251	—	6.1	5.5	—
2	0	—	269	269	—	5.65	5.65
3	0.1	331	260	—	6.15	5.55	—
4	0.1	297	275	—	5.85	5.7	—
5	0	286	275	—	5.8	5.7	—
6	0	—	275	275	—	5.7	5.7
7	0.1	295	269	—	5.85	5.65	—
8	0	295	260	—	5.85	5.55	—
9	0	297	260	—	5.85	5.55	—
Means	—	303.6 ± 16.9(7)	266.0 ± 8.6(9)		5.92 ± 0.14(7)	5.62 ± 0.079(9)	

of the detergent form with papain. As shown below, the two methods of exposure to proteinases generate forms that appear to be indistinguishable in all the properties so far examined.

Polyacrylamide-gel electrophoresis. The two forms had identical mobilities, unaffected by the presence or absence of 1% Triton X-100, in 7.7% polyacrylamide gels with the buffer system of Davis (1964). In the presence of dodecyl sulphate, both proteins migrated as polypeptides with an apparent subunit mol.wt. of about 130000. However, there was a consistent slight retardation of the detergent form (Plate 1*a*), but the difference in mobility was too small to permit resolution of the two forms, even when the loading was decreased to 2μg of protein. It is not possible to calibrate the difference in apparent subunit mol.wts., but since the system can easily resolve the large subunit of the papain form of aminopeptidase M (140000 mol.wt.) from dipeptidyl peptidase IV (130000 mol.wt.) (Booth & Kenny, 1976), it is likely that the difference between the two forms is small and probably in the range 3000–5000 mol.wt.

Sedimentation-velocity experiments. These were performed in single- (two runs) and double-sector (one run) cells at 21°C in 0.1M-Tris/HCl buffer, pH 7.9, at protein concentrations in the range 1.5–2.0mg/ml. The uncorrected sedimentation coefficients were in the range 9.6–10.25 for the detergent form and 10.25–10.45 for the autolysis form. Both forms exhibited single symmetrical schlieren peaks.

Gel filtration on columns of Sepharose 6B. The two forms were examined in the same sample, by exploiting the ease with which the enzyme could react with di-isopropyl [^{32}P]phosphorofluoridate. One form, the autolysis form, was detected by its radioactivity, whereas the detergent form was monitored by its enzymic activity. The results, shown in Table 3, revealed that, in buffers with and without Triton X-100, the autolysis form eluted in the same volume corresponding to a mol.wt. of 266000. The detergent form was consistently eluted in a smaller volume of buffer (mean mol.wt. 304000). In one experiment it was resolved from the autolysis form, in others it was only partially resolved. Again the presence or absence of detergent in the elution buffer did not influence the result. The detergent form appeared larger than the autolysis form by a rather variable amount. Both forms behaved as dimers, with a mean

Table 4. *Crossed immunoelectrophoresis of dipeptidyl peptidase IV*

The amount of enzyme added was in the range 1.5–2 μg of the purified forms. The detergent-solubilized microsomal fraction was treated with papain as described in the Methods section for the pure enzyme, except that 100 μg of papain/mg of protein was used. The second-dimension gel contained an immunoglobulin fraction from rabbits immunized with the detergent form of pig dipeptidyl peptidase IV.

Enzyme form	Enzyme (units) applied to gel	Electrophoretic mobility ($cm^2 \cdot V^{-1} \cdot h^{-1}$)
Detergent	20	0.06
Detergent, papain-treated	20	0.06
Autolysis	30	0.06
Detergent-solubilized microsomal fraction	30	0.04
Same after papain treatment	30	0.10

Table 5. *Crossed charge-shift immunoelectrophoresis of dipeptidyl peptidase IV*

The procedure was based on that described by Bhakdi *et al.* (1977) [see Booth *et al.* (1979) for details]. In the first dimension the agarose gel and buffers contained 0.5% (w/v) deoxycholate (DOC) or 0.0125% (w/v) cetyltrimethylammonium bromide (CETAB). The second-dimension gel contained an immunoglobulin fraction prepared from the serum of rabbits immunized with pig dipeptidyl peptidase IV (detergent form). The micosomal fraction was treated as described in Table 4.

Enzyme form	Enzyme (units) applied to gel	Electrophoretic mobility ($cm^2 \cdot V^{-1} \cdot h^{-1}$)	
		Triton/DOC	Triton/CETAB
Detergent	30	0.07	0.06
Detergent, papain-treated	30	0.07	0.06
Detergent-solubilized microsomal fraction	45	0.12	0.02
Same after papain treatment	45	0.08	0.10

difference in mol.wts. of 37600 and with individual differences in the range 11000–73000. Since the difference in apparent polypeptide subunit mol.wt. is about 3500, the larger difference observed on gel filtration must be attributed to bound detergent, adding, on the average, 30000 to the dimeric molecular weight. Papain treatment of the detergent form converted it into a form that co-eluted with the autolysis forms. The calculated Stokes radii of the detergent and autolysis forms were 5.9 and 5.6nm respectively.

Electron microscopy of the enzymes after negative staining with uranyl acetate. The appearance of both the detergent and autolysis forms shown in Plate 1(*b*) is that of a dimer, each subunit being about 7nm in diameter.

Immunoelectrophoresis. Detergent-solubilized microvillar enzymes have been shown to migrate more slowly in the first dimension of crossed immunoelectrophoresis than proteinase forms [see the following paper (Booth *et al.*, 1979)]. This difference, attributable to substantial amounts of bound detergent (Emulphogen BC720), was not detected when the purified detergent and autolysis forms of dipeptidyl peptidase IV were compared (Table 4). This unexpected result was not due to the use of Triton X-100 in place of Emulphogen BC 720. Furthermore, no change in mobility occurred in crossed charge-shift immunoelectrophoresis (Table 5). The technique (Helenius & Simons, 1977) exploits the ability of ionic detergents to associate with the non-ionic detergent bound to an amphipathic protein, thus conferring additional positive or negative charges to it, depending on the use of a cationic or anionic detergent. The failure to observe charge-shift with the purified enzymes contrasted with the positive results with an unpurified Triton X-100 extract of a microvillar fraction. A clear difference in mobility was observed between the detergent-solubilized activity and the same preparation after papain treatment. This different behaviour of the pure and crude forms of the detergent-solubilized enzyme is probably explained by the micellar form of the bound detergent in the crude form becoming depleted during enzyme purification, to an annulus of tightly bound detergent, not readily exchangeable with the ionic detergents.

Hydrophobic chromatography. Phenyl-Sepharose

CL-4B was capable of differentiating between the detergent and proteinase forms of the enzyme. Samples of the enzyme were applied to small columns (volume 0.5ml) of phenyl-Sepharose in 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100. Only 1.5% of the activity of the autolysis and papain forms bound to the column, compared with 31% of the detergent form. The same result was obtained with ^{125}I-labelled samples of the enzyme. Again 1% of the radioactivity of the papain form bound, whereas 35% of that of the detergent form bound and could be eluted by the addition of 50% (v/v) ethylene glycol to the buffer.

End groups of the detergent and proteinase forms identified by dansylation and carboxypeptidase Y. Dansylation of the urea-denatured reduced carboxymethylated derivatives of the two forms of the enzyme gave consistent results with different batches of the enzyme (Table 6). The detergent form had leucine as the *N*-terminal residue, whereas the autolysed form had serine; papain treatment of the detergent form generated a form of the enzyme having serine as the *N*-terminal residue.

Carboxypeptidase Y from baker's yeast caused extensive degradation of the detergent and papain forms to give a complex mixture of amino acids. It was not possible to identify the *C*-terminal amino acid residue among this complexity, but Table 7 illustrates the release of twelve amino acids in the approximate order of abundance as they appeared over the 3h period of incubation. The results show a general similarity in the pattern of release from the

Table 6. *N-Terminal amino acid residues identified by dansylation of the detergent and proteinase forms of dipeptidyl peptidase IV*

See the Methods section for details.

Enzyme	*N*-Terminal residue(s)	No. of preparations examined
Detergent form	Leucine	8
Autolysis form	Serine	4
Detergent form treated with papain	Leucine + serine	4

Table 7. *Release of amino acids from the detergent (D) form and the papain-treated detergent (P) form of dipeptidyl peptidase IV by carboxypeptidase Y*

See the Methods section for details. The results are given as mol. of amino acid released/130000g of protein.

Amino Acid	Enzyme form	Time of incubation (min) ...	Amino acid released 15	90	180
Leucine	D		0.28	0.86	1.6
	P		0.26	1.2	1.7
Tyrosine	D		0.18	1.2	1.4
	P		0.20	1.3	1.7
Valine	D		0.23	0.94	1.3
	P		0.22	0.89	1.2
Threonine	D		0.17	0.84	1.2
	P		0.22	1.0	1.4
Isoleucine	D		0.15	0.70	0.9
	P		0.14	0.78	1.1
Serine	D		0.12	1.02	0.95
	P		0.12	0.77	0.96
Glutamic acid	D		0.26	0.67	0.86
	P		0.10	0.66	0.85
Alanine	D		0.30	0.67	0.64
	P		0.23	0.59	0.88
Phenylalanine	D		0.17	0.56	0.76
	P		0.10	0.60	0.82
Histidine	D		0.06	0.58	0.83
	P		0.06	0.66	0.94
Aspartic acid	D		0.04	0.22	0.40
	P		0.13	0.44	0.55
Methionine	D		0	0.22	0.32
	P		0	0.14	0.22

detergent and papain forms of the enzyme. The evidence presented indicates that the two polypeptides probably have the same sequence at the *C*-terminus while possessing clearly different *N*-termini.

Amino acid sequences of the N-terminal regions of the detergent and autolysed forms of the enzyme. The difference in *N*-terminal residues of the two forms extended to other amino acid residues in this region of the sequence. The autolysed form was sequenced in duplicate samples by a spinning-cup automatic sequencer to yield a partial identification of the first 16 residues, five or six of which were hydroxy amino acids (Fig. 1). For reasons as yet unexplained, the detergent form could not be sequenced automatically, in spite of four attempts. A blocked *N*-terminus was excluded as the cause of this failure, and indeed it was possible to obtain a short sequence by manual Edman degradation. The first five residues have been confirmed in duplicate degradations. The sequence of the first eight residues contrasts with that of the autolysis form in being strongly hydrophobic in character.

Analysis of the carbohydrate in the detergent and papain forms of the enzyme. These results are shown in Table 8. Glucose and fucose show slight differences between the two samples analysed, but in general the content of sugars is very similar for the two forms of the enzyme.

Isolation of a hydrophobic peptide after treatment of the detergent forms with papain

It has been shown above that papain converts the detergent form into one that is indistinguishable from the autolysis form. This proteinase form had a different *N*-terminus, had a slightly lower apparent subunit molecular weight, and behaved as a hydrophilic rather than an amphipathic protein. We have fractionated the papain digest of the detergent form in order to characterize the hydrophobic peptide that was presumed to have been cleaved from the enzyme.

Fractionation of the papain digest of the detergent form. The scheme involving gel filtration and ion-exchange chromatography using buffers containing Triton X-100 is shown in Scheme 2 (above). Sephadex G-50 resolved larger peptides from very small fragments and free amino acids. The former fraction was then resolved into bound (I) and unbound fractions (II) by DEAE-cellulose and each was further resolved by Sephadex G-150. Five peptide and protein fractions were studied. Two were identified as the hydrophilic form of dipeptidyl peptidase IV (fraction Ia) and papain (fraction IIb) respectively. The other three were oligopeptides, designated as fractions Ib, IIa, IIc. A sample of the autolysis form of the enzyme was similarly treated with papain and fractionated.

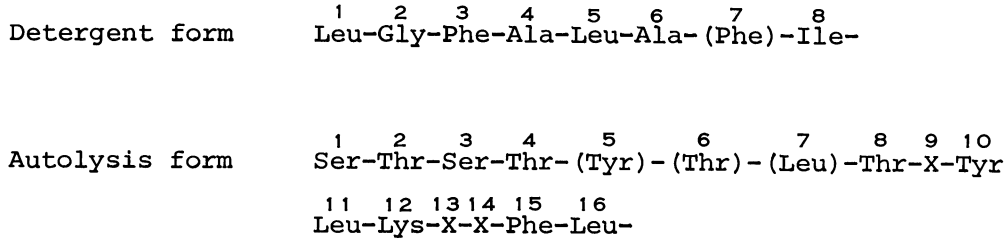


Fig. 1. *Amino acid sequences of the N-terminal regions of the detergent and autolysis forms of dipeptidyl peptidase IV* See the Methods section for details. Each sequence is the result of two degradations. Parentheses denote a tentative assignment for a residue.

Table 8. *Carbohydrate analysis of the detergent and proteinase forms of dipeptidyl peptidase IV*
See the Methods section for details. The proteinase form was resolved from papain and other products by chromatography on Sephadex G-150. The results are expressed as mol/130000g of protein.

Sugar residue	Content: Detergent form	Content: Detergent form after papain treatment
Glucose	13.6	7.2
Galactose	37.0	35.5
Mannose	18.5	20.5
Fucose	5.2	12.4
N-Acetylgalactosamine	27.3	25.7
N-Acetylglucosamine	6.1	7.9

Sephadex G-50 resolved two comparable fractions: an excluded peak of enzyme activity and some low-molecular-weight material. All the enzyme activity bound to DEAE-cellulose which, when eluted and run on Sephadex G-150, appeared in fraction Ia. The unbound material yielded a fraction corresponding to IIb (papain), but no fractions corresponding to Ib, IIa and IIc. The columns were monitored by fluorescamine; samples of the fractions were also hydrolysed in 10M-NaOH to ensure that no peptides escaped detection by fluorescamine.

The retarded fraction from the first column, Sephadex G-50, was examined by high-voltage paper electrophoresis. All the ninhydrin-positive components migrated towards the cathode at pH1.85. Part of this fraction had been labelled with ^{125}I by using the Bolton & Hunter (1973) reagent. Radioautography did not reveal any slow-moving or immobile radioactive components. This fraction appeared, therefore, to contain only free amino acids and very small peptides. It was not further investigated. The excluded peak contained all the dipeptidyl peptidase IV activity and any other materials of mol.wt. greater than about 9000. On DEAE-cellulose the enzyme activity bound and was eluted by increasing the ionic strength of the buffer in Fraction I. Any material not bound (fraction II) was concentrated by freeze-drying. Both fractions were further resolved on columns of Sephadex G-150. fraction I yielded an excluded peak (fraction Ia) that was identified as the hydrophilic form (papain form) of the enzyme, since it contained the enzyme activity and was shown by dansylation to have serine as the *N*-terminal residue. A retarded fluorescamine-positive peak was designated Ib. Fraction II was resolved into three fluorescamine-positive peaks: an excluded peak (IIa), another peak (IIb) with an elution volume corresponding to that previously determined for papain, and a third of lower molecular weight, designated IIc. Portions of fractions Ib, IIa and IIc were labelled with ^{125}I by using the Bolton & Hunter (1973) reagent in order to simplify the recognition of the components in subsequent analyses. Fraction IIb was shown to be single-banded on polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate; the mobility of the polypeptide was consistent with its identification with papain.

Polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate. A 20% polyacrylamide gel was prepared and run by using the discontinuous system devised by Booth (1977). Fractions Ib, IIa and IIc were run in parallel tracks, together with some ^{125}I-labelled marker polypeptides. After staining and destaining, no bands were visible in the tracks containing the unknown fractions, and relatively little of the applied radioactivity remained. However duplicate tracks of the same slab were dried and subjected to radioautography without preliminary staining. In this case each of the three fractions Ib, IIa and IIc revealed, after 36h exposure, a single radioactive band with a mobility similar to that of insulin B-chain (Plate 2). Very much longer exposure (21 days) of the stained half of the gel slab showed radioactive bands with the same mobilities. It appeared that the peptides were not adequately fixed during staining and most of the material was lost during the staining and destaining cycles. Each of the three fractions therefore contained a peptide of about 3500 apparent mol.wt., in spite of their different behaviour in previous steps. Fraction IIa had eluted from Sephadex G-150 with an elution volume only slightly greater than that of the papain form of dipeptidyl peptidase IV (mol.wt. 260000). Fractions Ib and IIc had eluted as smaller complexes and with similar elution volumes, but the column was not sufficiently well calibrated to define their molecular size. However, it is probable that the differences in molecular size observed in gel filtration arose from the binding of detergent and, in the case of fraction IIa, to extensive aggregation of the peptide.

Fractionation of peptides Ib, IIa and IIc in chloroform/methanol mixtures. Samples of the three peptide fractions were partitioned between the aqueous and organic phases of chloroform/methanol mixtures. The peptide had been ^{125}I-labelled by the Bolton & Hunter (1973) reagent. A similarly labelled sample of insulin B-chain was also studied; it partitioned (92%) into the aqueous phase. In contrast, the radioactivity of each of the peptide fractions partitioned abundantly (64%) in the organic phase.

Hydrophobic chromatography on phenyl-Sepharose CL-4B. Labelled samples of the peptides and insulin B-chain in 5mM-Tris/HCl (pH7.9)/0.1% Triton X-100, were loaded on to columns of phenyl-Sepharose. Only 6% of insulin B-chain radioactivity bound to the columns, compared with 78–85% of the radioactivity of the three peptide fractions. About 75% of the bound radioactivity was recovered by elution with 50% (w/v) ethylene glycol.

Identification of the N-terminal amino acid residues by dansylation and amino acid analyses of the peptides. Each of the peptides Ib, IIa and IIc were shown to have leucine as the *N*-terminal residue. The amino acid analyses (Table 9) were generally similar for the three peptides. An assumed mol.wt. of 3500 was used in presenting the results. On this basis the peptide contains 28–30 amino acid residues, of which eight or nine are unequivocally hydrophobic, eight or nine unequivocally polar, with the remainder including glycine, alanine and Glx (glutamic acid/glutamine). The polarity index (Capaldi & Vanderkooi, 1972) was 41% for each fraction, though if all four Glx residues were glutamine, the index would be slightly lower, about 36%. The value for tryptophan is an

underestimate of the true content; if known, this would also decrease the polarity index.

Discussion

The object of the present work was to purify dipeptidyl peptidase IV in the form in which it exists in the intact membrane. The oligomeric state of this, or, for that matter, other enzymes in the microvillar membrane, has not been elucidated. The dimeric state of dipeptidyl peptidase IV when purified after proteinase or detergent solubilization makes it very probable that it occurs in dimeric form (or possibly multiples of dimers) in the membrane. If the dimerization were an artifact of preparation, one might expect the production of a polydisperse population including mixed dimers of dissimilar subunits. A more important consideration than the oligomeric state is that the primary structure of the purified detergent form should be identical with the native enzyme. This is difficult to prove, since it is impossible to rule out the presence of an endogenous proteinase causing limited proteolysis during the early steps in fractionation. The apparent subunit molecular weight of the ^{32}P-labelled polypeptide, estimated by dodecyl sulphate / polyacrylamide - gel electrophoresis, in purified microvillar fractions is not significantly different from that of the purified enzyme (Kenny *et al.*, 1976). But it might still be argued that proteolysis had already occurred during subcellular fractionation. In the case of some intestinal microvillar enzymes, e.g. aminopeptidase M, it has been suggested that contact with pancreatic proteinases *in vivo* precludes the isolation of an enzyme without some limited proteolytic degradation (Sjöström *et al.*, 1978). However, the kidney does not present problems of this type. Endogenous proteinases capable of endopeptidase activity seem to be limited to those in the lysosomes, which, in the case of dipeptidyl peptidase IV, require prolonged incubation at low pH values to release the enzyme from the membrane in high yield. In one of the early preparations of the detergent form of the enzyme (not described in the Results section) we took steps to prevent proteolysis during the purification. The tissue was homogenized in the presence of antibacterial agents (streptomycin and penicillin). A portion (15%) of the microsomal fraction was incubated with di-isopropyl [^{32}P]-phosphorofluoridate and added back to the bulk of the fraction, which now contained 1 mM-di-isopropylphosphorofluoridate. The enzyme was then purified as a ^{32}P-labelled protein. Its behaviour on Sepharose 6B and on analytical ultracentrifugation was the same as in subsequent preparations. On another occasion the early steps of the purification were carried out in 1 mM-phenylmethanesulphonyl fluoride

Table 9. *Analyses of amino acids of three hydrophobic peptides (Ib, IIa and IIc) from the treatment of the detergent form of dipeptidyl peptidase IV with papain*

See the Methods section for details of the fractionation. Samples were hydrolysed in tubes sealed under N_2 with 5.7 M-HCl containing a crystal of phenol for 24h at 110°C. The values are expressed as mol of amino acid residue/3500g of peptide.

	Content (mol of amino acid/3500g of peptide)					
	Fraction ... Ib		IIa		IIc	
Amino acid	Observed values	Next integer	Observed values	Next integer	Observed values	Next integer
Ala	1.34	1	2.74	3	1.57	2
Arg	—	—	—	—	—	—
Asx	—	—	—	—	—	—
Cys	—	—	—	—	—	—
Glx	4.08	4	3.94	4	4.11	4
Gly	5.85	6	6.91	7	6.48	6
His	0.6	1	0.54	1	0.68	1
Ile	1.31	1	1.31	1	1.46	1
Leu	2.4	2	1.97	2	2.28	2
Lys	0.91	1	0.51	1	0.8	1
Met	—	—	—	—	—	—
Phe	0.6	1	0.97	1	0.8	1
Pro	—	—	—	—	—	—
Ser	5.0	5	4.54	5	4.43	4
Thr	1.48	1	1.91	2	1.74	2
Trp	2.34	2	0.71	1	1.31	1
Tyr	0.74	1	0.94	1	0.88	1
Val	1.86	2	1.48	1	1.91	2

(which did not significantly inhibit dipeptidyl peptidase IV) as well as penicillin, streptomycin and NaN_3. Again the properties, including the identity of the *N*-terminal amino acid residue, were unchanged. The consistency with which leucine was identified as the *N*-terminal amino acid residue in every batch of detergent-solubilized enzyme subjected to dansylation, argues against the occurrence of random attack by endogenous proteinases during extraction and purification.

Svensson *et al.* (1978) have recently reported the purification of a Triton X-100-solubilized form of dipeptidyl peptidase IV from pig intestine. Its apparent subunit mol.wt. was about 137000, but its mol.wt. by gel filtration was 230000. Since it bound a substantial amount of detergent, as shown by charge-shift crossed-immunoelectrophoresis, this result might indicate that the intestinal enzyme is monomeric.

Hydrophobic domain of the detergent form of dipeptidyl peptidase IV. If we are right in our assumption that the detergent-solubilized enzyme has the same primary structure as the native enzyme, we can now consider the evidence that the detergent form differs from the autolysis form in possessing an extension of the polypeptide chain that serves to anchor the protein in the lipid bilayer of the membrane.

First, we have consistently observed a slight retardation in mobility of the detergent form of the enzyme compared with the autolysis form on electrophoresis in the presence of dodecyl sulphate. The difference was small and we were unsuccessful in our attempts to resolve mixtures of the two forms present in the same sample. Differences in the extent of glycosylation of the two forms can be excluded as an explanation, since the analyses of sugars were essentially similar. It is difficult to quantify the difference in apparent molecular weights, but we know that a difference of 10000, e.g. that between dipeptidyl peptidase IV and the papain-released form of aminopeptidase M, can be easily resolved in this system (Booth & Kenny, 1976). The difference in molecular weight between the detergent and autolysis forms of the enzyme is probably in the range of 2500–5000.

Secondly, the two forms can be partially, and in some experiments almost completely, resolved by gel filtration in columns of Sepharose 6B. This difference cannot be attributed solely to the relatively small extension of the polypeptide chain. Rather it indicates that this additional domain of the protein binds significant amounts of Triton X-100. The calculated molecular weight for the autolysis form was relatively consistent, 266000 ± 8600 (S.D.). The detergent form gave values in the range 286000–331000, with a mean of 303600 ± 16900 (S.D.). Since the two forms were always examined concurrently, there is no reason why the error of the method should be greater for the detergent form. We interpret the results as indicating that the amount of detergent bound is variable. If the two hydrophobic peptide domains account for a difference of, say, 7000 in mol.wt., the bound detergent might contribute a further 4000–66000 to the difference. The monomeric mol.wt. of Triton X-100 is 640 (Robinson & Tanford, 1975), so that the amount of bound detergent appears to be in the range 8–100 (with a mean of about 50) molecules to each dimer. A Triton X-100 micelle contains 100–150 molecules of detergent (Helenius & Simons, 1975). Most of our samples of enzyme bound much less than this number. The difference in behaviour of freshly solubilized microvillar membrane proteins compared with the purified enzyme in crossed immunoelectrophoresis supports the view that dipeptidyl peptidase IV is initially released associated with a detergent micelle, but that detergent is progressively shed during enzyme purification until a minimal quantity, perhaps an annulus of detergent, remains associated with the relatively small hydrophobic domain. Whatever may be the quantity and state of organization of the bound detergent, the evidence for its presence in the detergent form of the enzyme demonstrates that this form possesses a hydrophobic domain absent from the proteinase form. Many membrane enzymes purified in their detergent forms undergo aggregation if detergent is omitted from the media used in their purification. This phenomenon has been reported for several microvillar enzymes, e.g. intestinal aminopeptidase M (Maroux *et al.*, 1973), γ-glutamyl transferase (Hughey & Curthoys, 1976), intestinal dipeptidyl peptidase IV (Svensson *et al.*, 1978) and intestinal sucrase–isomaltase (Semenza, 1978). Kidney dipeptidyl peptidase IV was anomalous in remaining dimeric if detergent was omitted from the later steps of purification, although omission of detergent early in the purification precluded effective recovery from columns, presumably because of aggregation of the enzyme. Although we cannot exclude loss of part of the hydrophobic domain with its attendant detergent to account for this alteration in behaviour, we favour the view that the change in size and susceptibility to aggregation were the results of depletion of the original micelle to a state in which only an obligatory minimum number of tightly bound detergent molecules protected the hydrophobic domain from engaging in other hydrophobic interactions. The observation that the detergent form displayed hydrophobic properties in binding to phenyl-Sepharose CL-4B supports this opinion.

Thirdly, the non-identical amino acid sequences of the *N*-terminal regions of the autolysis and detergent forms confirms a difference in primary structure. The sequence has not been elucidated for a sufficient number of residues for us to predict with confidence that the hydrophobic domain of the native enzyme

extends to the *N*-terminus, but there is a notable lack of polar amino acids among the eight so far identified. It would be desirable, though this has not yet proved possible, to extend the sequence of the detergent form to a region where it overlaps with the proteinase form, an experiment that might require a further 30 amino acid residues to be identified.

Fourthly, treatment of the detergent form with papain generated a hydrophilic form of the enzyme indistinguishable in properties from the autolysed form. In the process a fragment was formed that had distinctly hydrophobic properties. The characteristics of this peptide are further discussed below.

The evidence presented above establishes that the detergent form differs in primary structure from the proteinase form in its possession of a hydrophobic domain. The only negative findings are those, already discussed, concerning immunoelectrophoresis and the identical sedimentation coefficients of the two forms. This latter result is not a serious argument against the main conclusion. The extension of the polypeptide in the detergent form probably contributes only about 3500 to the monomeric molecular weight, and this may be counterbalanced by the buoyancy effect of the bound detergent.

The hydrophobic peptide liberated by papain treatment of the detergent form

Papain had only one demonstrable effect on the detergent form of the enzyme, namely that of conferring on the enzyme all the properties noted for the autolysis form. Catalytic activity was unchanged and the new form was structurally indistinguishable from the autolysis form in respect of its *N*-terminal residue, serine, and its elution volume on gel filtration. Since the position of the X–Ser bond cleaved by papain has not been determined, nor that by the endogenous lysosomal proteinases active during autolysis, it would be premature to assume that the same bond has been cleaved in both cases.

Apart from a fraction containing some free amino acids and possibly some small peptides (the origin of which are uncertain, but were probably derived from autodigestion of the papain since they appeared in the control incubation with the autolysis form), the only detectable peptide fragments in the digestion mixture appeared in three fractions resolved by ion-exchange chromatography and gel filtration. Their difference in behaviour during fractionation appeared to be the result of differing extents of aggregation and binding of detergent. The apparent subunit size of the peptide in each fraction was about 3500 mol.wt., each had leucine as the terminal residue and each had a similar content of amino acids on hydrolysis. We conclude that the same peptide was present in the three fractions. This peptide is derived from that part of the native enzyme that includes the hydrophobic domain serving to anchor each subunit to the membrane. This conclusion is based on the following arguments. First, the fragment is of a size consistent with the difference in apparent subunit molecular

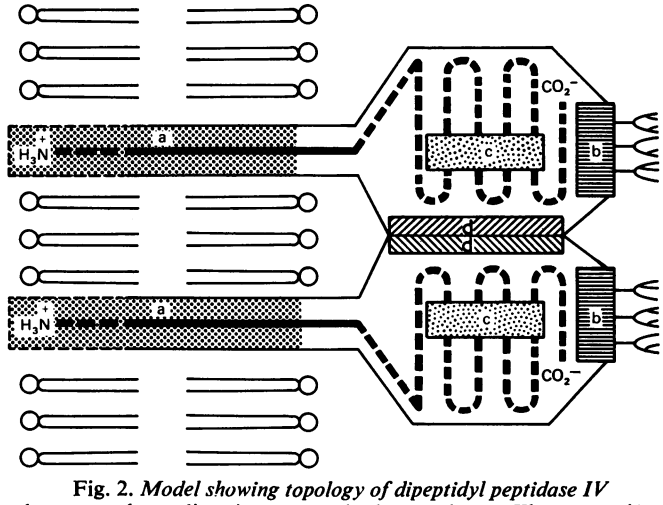


Fig. 2. *Model showing topology of dipeptidyl peptidase IV*

The model assumes that the enzyme has a dimeric structure in the membrane. The uncertainty concerning the extent of penetration of the anchor across the membrane is indicated by the broken lines near the internal surface of the membrane. Four domains on each subunit are represented schematically: (a) a surface participating in association of the two monomers; (b) a glycosylated region; (c) a catalytic site containing the reactive serine residue and; (d) a hydrophobic domain, at or near the *N*-terminus, anchoring the protein to the lipid bilayer. The model is not to scale; the anchor comprises no more than 2–3% of the polypeptide chain.

weights of the two forms, and there is therefore no reason to believe that any other substantial fragments were lost during fractionation. Secondly, the peptide had undoubted hydrophobic properties; it extracted preferentially into chloroform/methanol, bound strongly to phenyl-Sepharose CL-4B, was extracted rather than fixed by propan-2-ol/acetic acid mixtures used in the staining of polyacrylamide gels after electrophoresis and was capable of aggregation and binding variable amounts of detergent. However it should be noted that the amino acid composition had a polarity index of 41%, indicating that the peptide contained hydrophilic as well as hydrophobic regions.

Topology of dipeptidyl peptidase IV

A model for this enzyme that is consistent with the experimental results is that shown in Fig. 2. It is that of a dimer comprising identical monomers of about 130000 mol.wt. and each containing four domains. These domains are: (*a*) a surface participating in the association of the two monomers to form the dimer and involving interactions yet to be determined, but probably not of a hydrophobic nature, since non-ionic detergents do not dissociate the subunits, and not involving disulphide bonds, since omission of 2-mercaptoethanol in the preparation of samples for electrophoresis in dodecyl sulphate did not prevent the protein migrating as subunits (A. G. Booth, unpublished work); (*b*) a glycosylated region; (*c*) a catalytic site in which the reactive serine residue is located; and (*d*) a hydrophobic domain associated with the membrane.

Only (*d*), the anchor, can be located with confidence to a region at or near the *N*-terminus of the polypeptide chain, somewhere within the first 30–40 amino acid residues. It may indeed extend to the leucine residue at the *N*-terminus; the eight residues so far identified in the sequence are non-polar and, if these are subtracted from the amino acid composition of the papain-released peptide, the remaining amino acids include many that are relatively polar in nature and unlikely to provide a second hydrophobic sequence more remote from the *N*-terminus. The implication is that the anchor may be very small, 15–20 amino acid residues.

The assumption that the anchor is close to the *N*-terminus is based on the non-identity of the amino acid sequences adjacent to the *N*-termini of the detergent and autolysis forms, coupled with the evidence, from digestion with carboxypeptidase Y, that the *C*-terminal regions of the two forms are probably identical. A model in which the *C*-terminal region is involved in anchoring the protein would require a more complex series of assumptions. It would be necessary to postulate an attack by papain at both ends of the polypeptide chain: (*a*) near the *C*-terminus to liberate a hydrophobic peptide with an *N*-terminal leucine and at the same time exposing a new *C*-terminal sequence of the enzyme that closely resembles that in the native enzyme (i.e. requiring some internal homology in the sequence); and (*b*) near the *N*-terminus to generate an *N*-terminal serine and liberating a leucine *N*-terminal fragment. We prefer the simpler explanation for the data, as summarized in our model, though it must be admitted that its unequivocal proof would require a more detailed investigation of the primary structure, including the sequencing of substantial fragments.

Is it heresy to postulate that the N-terminal region of a membrane protein is associated with the lipid bilayer?

Dipeptidyl peptidase IV is, in the terminology of Rothman & Lenard (1977), a typical ectoprotein. The bulk of the protein, including the glycosylated and active-site domains, protrudes from the external surface of the membrane. Such proteins are thought to be synthesized by membrane-bound polyribosomes, a view that is supported by work on the glycoproteins of vesicular-stomatitis and sindbis viruses (Morrison & Lodish, 1975; Wirth *et al.*, 1977). Their biosynthesis is thus analogous to that of secreted proteins, in which the attachment of the polyribosomes to the membrane is dictated by the signal sequence at the *N*-terminus of the nascent polypeptide chain and by a receptor protein in the membrane (for review, see Campbell & Blobel, 1976). The signal peptide is typically about 20 amino acid residues in length, often hydrophobic in character (see e.g. Habener *et al.*, 1978), and is removed by signal peptidase before synthesis of the protein is completed. The glycoprotein of vesicular-stomatitis virus is assembled asymmetrically in the membrane of the endoplasmic reticulum (Katz *et al.*, 1977) in such a manner that the bulk of the protein, including the *N*-terminus and the glycosylated domain, is within the cisternal space. The *C*-terminal region of the polypeptide (about 5% of the protein) is exposed at the cytoplasmic surface of the membrane, with the hydrophobic anchor located in the sequence between these two domains. When, by membrane fusion, the glycoprotein is assembled in the plasma membrane, the *C*-terminus remains on the cytoplasmic surface and the bulky glycosylated domain now protrudes from the external surface of the cell. Rothman & Lenard (1977) have suggested that this is a general model for the biosynthesis and assembly of ectoproteins. The topography of several ectoproteins appears to conform to this model. Glycophorin in erythrocytes is the best example. The polypeptide chain contains 131 residues (Tomita & Marchesi, 1976); residues 1–72, including those that are glycosylated, are external, the hydrophobic anchor region occupies residues 73–92 and the remaining 38 residues are on the cytoplasmic surface. The histocompatibility antigens, mouse H2 (Henning *et al.*,

Table 10. *Molecular properties of detergent- and proteinase-released forms of some microvillar enzymes*
–, Information not available. References: 1, Maroux *et al.* (1973); 2, Maroux & Louvard (1976); 3, Wacker *et al.* (1976); 4, Booth & Kenny (1976); 5, Vannier *et al.* (1976); 6, Hughey & Curthoys (1976); 7, the present paper; 8, Semenza (1978), 9, Colbeau & Maroux (1978).

			10^{-3} × Molecular weight		'Detergent' and 'proteinase' forms		
Enzyme	EC no.	Source	Membrane (detergent form) subunit(s)	Anchor	*N*-Terminal residue(s)	*C*-Terminal residue(s)	References
Aminopeptidase M	3.4.11.2	Intestine	130, 97, 49	9	Different	—	1,2
Aminopeptidase M	3.4.11.2	Kidney	160	10	Different	—	3,4,5
γ-Glutamyltransferase	2.3.2.2	Kidney	27 + 54	3	—	—	6
Dipeptidyl peptidase IV	3.4.14.–	Kidney	130	4–5	Different	Same	7
Isomaltase	3.2.1.10	Intestine	140	17	Different	Same	8
Sucrase	3.2.1.48	Intestine	120	0	Same	Same	8
Alkaline phosphatase	3.1.3.1	Intestine	64	4	Same	—	9
Maltases		Intestine	—	8–10	Different	—	2

1976) and human HLA (Springer & Strominger, 1976) and the influenza virus haemagglutinin (Skehel & Waterfield, 1975) are also anchored to the membrane by a region at or near the *C*-terminus.

Our results for dipeptidyl peptidase IV in the kidney microvillus membrane are inconsistent with this topological model. Our conclusion, that the protein subunits are anchored by the *N*-terminal region, requires a different mode of assembly in the membranes of the endoplasmic reticulum. The *N*-terminal region of the nascent polypeptide chain must somehow re-insert into the membrane from the cisternal side. This prompts the speculation that the hydrophobic anchor might be the signal peptide which, in this instance, is immune from attack by signal peptidase. Thus the signal peptide serves both its vectorial function at the start of biosynthesis and then an anchoring function for the finished polypeptide. Whether or not this speculation is correct, it is now necessary to accept that the orientation of proteins, such as glycophorin, is not a model for all ectoproteins. Other microvillar membrane proteins share common topological features with kidney dipeptidyl peptidase IV. Table 10 summarizes the information on the relative size and, where details are known, the orientation of the polypeptide chain in the membrane (see Table 10 for references). The isomaltase subunit of the sucrose–isomaltase complex in the rabbit intestine has been partially sequenced from the *N*-terminus. Residues 10–31 are strongly hydrophobic and probably represent the anchor region within the lipid bilayer. The *C*-terminal amino acids are apparently similar for the detergent and proteinase forms. Information is less complete for aminopeptidase M in intestine and kidney and for two intestinal disaccharidases that hydrolyse maltose, but in each case the *N*-terminal amino acid residue differs in the detergent and proteinase forms. Curiously, alkaline phosphatase appears to have the same first and second amino acid residues, but it is not known if the *C*-terminal sequences are similar or different for the two forms. Sucrase is atypical in that this subunit in the sucrase–isomaltase complex lacks a hydrophobic anchor; it is attached only to the isomaltase subunit. The weight of evidence from these examples is that a class of membrane ectoproteins exists, at least in the microvillar membranes of kidney and intestine, in which the proteins are anchored to the membrane by a relatively small domain at or near the *N*-terminus.

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