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RABBIT INTESTINAL AMINOPEPTIDASE N

PURIFICATION AND MOLECULAR PROPERTIES

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Summary

The detergent and protease forms of rabbit intestinal aminopeptidase N were purified for chemical investigations and future specific immunological labeling of the enzyme in situ. The purification of the detergent form required a special technique called 'reverse immunoabsorbant chromatography'. The specific activity of the detergent form finally obtained was identical to that of the protease form. A significant charge micro heterogeneity persisted in the most purified preparations, due probably to a certain level of variability in the sugar moiety. The major proteolytic cleavage which occurred at the hydrophilic-hydrophobic junction of the detergent form during its conversion into the protease form was well defined. But additional splittings probably in the C-terminal region of the molecules led to several protease forms differing by their size.

The molecular weight assigned to the peptide liberated during the above conversion was overestimated due to preferential detergent binding to hydrophobic structures. The correct value, estimated by a new isotopic dilution method, was 3800 (36—38 residues) for the peptide originating from the rabbit enzyme. The real anchor plunging into the membrane core is possibly still shorter.

Comparative N-terminal residue determinations in the detergent form, the protease form and the peptide definitely confirmed that the enzyme is anchored to the bursh border membrane by its N-terminal region.

Introduction

The brush border membrane of pig enterocytes has been reported several years ago to contain substantial amounts of an aminopeptidase active on

neutral peptides and synthetic substrates [1]. It has more recently been realized that another aminopeptidase highly active on acidic amino acid derivatives also exists in the brush border [2,3]. It is called aminopeptidase A (for acidic). We suggest to call the first one, aminopeptidase N (for neutral) rather than aminopeptidase M (microsomal) as proposed for the kidney enzyme [4]. In fact, the renal and intestinal aminopeptidases are not localized in the endoplasmic reticulum, but in the brush border which co-sediments with the microsomes after vesiculization.

Topological studies have shown that pig intestinal aminopeptidase N is a transmembrane protein composed of a hydrophilic, sugar-rich and enzymatically active 'head' protruding from the external face of the membrane and of a short, predominantly hydrophobic anchor spanning the lipid core [5—7]. Like several other membrane enzymes, the entire aminopeptidase molecule is extracted by neutral detergents leading to an amphipatic form called the detergent form. The enzyme can also be solubilized by proteolytic cleavage at the hydrophilic-hydrophobic junction of the protein chain. The resulting form roughly corresponding to the hydrophilic head [5] and freely soluble in water is known to arise from papain digestion of intact brush border membrane, trypsin or papain digestion of the previously extracted detergent-form [8] and from spontaneous degradation by endogeneous protease(s) of the detergent-form during its purification. Although the resulting molecules are not identical, all will be referred to in what follows as the protease form, irrespective of the protease actually involved in the splitting.

The pig represents a rich and convenient source of intestinal enzymes for biochemical investigations. However, a smaller animal appears to be more appropriate for studies now in progress concerning the biosynthesis of these enzymes, their intracellular transport and mode of insertion into the membrane. Although rat aminopeptidase N has recently been purified [9,10], the rabbit enzyme was selected for several reasons to be discussed later. A technique for the purification of rabbit aminopeptidase has already been reported [11]. But, the results obtained during the last step of the procedure were not completely clear and only the protease form of the enzyme was purified.

The purpose of this study was to work out a convenient technique for the purification of the two forms of rabbit intestinal aminopeptidase N. The protease form was purified by conventional techniques. By contrast, full purification of the detergent form required the use of a special 'antiimpurities immunoabsorbant chromatography'. The finding that both forms are monomeric facilitated the investigations on the composition, exact size and position of the hydrophobic anchor.

Materials and Methods

Material

DEAE-cellulose (microgranular grade DE 32) was from Whatman, Sepharose 6-B and Phenyl Sepharose CL-4N from Pharmacia, Ultrogel AC A 34, Ultrogel AC A 22 and Concanavalin A — Ultrogel from Industrie Biologique Française. Hydroxyapatite and Agarose (Standard low-mr) were Biorad products. L-Alanine *p*-nitroanilide and α-L-glutamic acid *p*-nitroanilide were obtained,

respectively, from Bachem and Merck. Phenyl methyl sulfonylfluoride was from Sigma, Benzamidine-hydrochloride from Fluka and Emulphogen BC 720 from GAF.

Buffer A containing 10 mM monopotassium phosphate adjusted to pH 6.0 with 0.1 M KOH. Buffer B was a mixture of 10 mM dipotassium phosphate and 0.15 M NaCl adjusted to pH 7.4 with phosphoric acid.

Physicochemical methods

The preparation of purified brush border vesicles [12], the solubilization of the enzymes by detergents or proteases [8], the determination of enzymatic activities, sugar estimation, N-terminal residues identification and polyacrylamide gel electrophoresis were performed as previously described [1,5,8]. Glucosamine, galactosamine and amino acids were estimated with an automatic amino acid analyzer Beckman Model 120 C. The results obtained after 4, 10 and 24 h hydrolysis were extrapolated to zero time. Total proteins were estimated spectrophotometrically at 280 nm using $E^{1\%}_{1cm} = 13.5$ for the extinction coefficient of purified aminopeptidase. Zn content was determined using a Perkin-Elmer 300 atomic absorption spectrophotometer.

All centrifugation assays were carried out in a Spinco-Beckman ultracentrifuge Model E. The protein samples were dialyzed before use for 24 h against buffer A containing 0.15 M NaCl. The absorption coefficient $E^{1\%}_{1cm}$ of the protease form (13.5) was evaluated with 2 mg/ml protein solutions using the interferential optics of the Ultracentrifuge. Sedimentation coefficients were determined at 20°C and 60 000 rev./min. The apparent molecular weights were measured by the equilibrium method of Yphantis [13] on 1 mg/ml samples at 16 000 rev./min for 28 h at 20°C. Partial specific volumes were evaluated with the aid of a carefully thermostated microdensimeter (A. Parr; Model 120 C). In each run with this latter technique, several assays were realized at various protein concentrations. The results were considered correct when the plot of $\overline{V}$ versus protein concentration (including zero concentration) was linear.

Immunization and purification of the immunoglobulins from immunsera

Guinea pigs were immunized with 3.0 mg of Emulphogen extracted brush border proteins [5,8]. One third of this antigenic material in 500 μl of buffer B was emulsified with the same volume of complete Freund's adjuvant and injected subcutaneously (3—5 shots) into the neck of the animals. A second third was injected intramuscularly after 1 week followed by the injection of one sixth of the total material after 10 days. A last injection was made 1 day later with the rest of the material. The animals were bled 1 week after the last injection. For goat immunization, 9 mg of antigen (protease form or total impurities) were divided into 3 equal fractions. The first was emulsified with the same volume of complete Freund's adjuvant and injected subcutaneously (10 shots) into the back of the animal. Three weeks later, the second fraction was emulsified in the same volume of incomplete Freund's adjuvant and injected intramuscularly. The third fraction was injected subcutaneously (4—5 injections) 10 days later. The goat was bled 10 days after the last injection.

In both cases, the immunoglobulins were purified from the sera by ammonium sulfate precipitation and DEAE-Sephadex chromatography [14]. They were stored at 4°C in buffer B containing 0.1% sodium azide.

Double immunodiffusion

Experiments were carried out in a 0.9% (w/v) agarose gel swelled in a Veronal buffer at pH 8.2.

Crossed immunoelectrophoresis

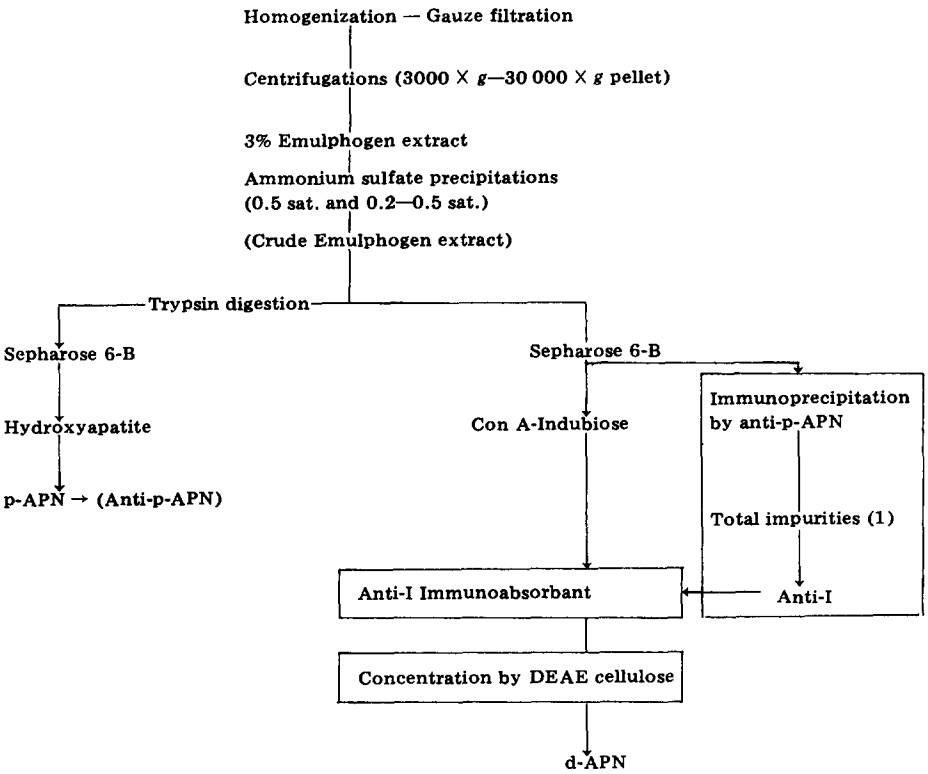
Crossed immunoelectrophoresis [15,16] was performed in a Holm-Nielsen apparatus. Ten ml of 1% (w/v) agarose gel in a 37.5 mM Tris-0.1 M glycine buffer (pH 8.7) containing 0.3% NaN_3 and 1% (v/v) of Emulphogen were layered on glass plates (100 mm × 100 mm). The same buffer without Emulphogen was placed in the electrode compartments. Electrophoresis was carried out at 5 V/cm for 1 h in the first dimension and at 1 V/cm for 18 h in the second. The excess of immunoglobulins used for the second dimension was removed with a 1-cm thick layer of soft cellulose tissue maintained for 10 min at a pressure of about 10 g/cm^2. The gel was rehydrated in phosphate buffered saline and distilled water for 10 min and pressed again. Specific stainings of enzymatic activities were performed as previously described for polyacrylamide gels [8]. For protein staining, the gels were dried in a stream of warm air. The plates were immersed for 4 min into a 0.25% Coomassie Blue solution [8] and destained in ethanol/acetic acid/water (25 : 10 : 45, v/v). In the rocket immunoelectrophoresis, the electrophoresis in the first dimension was omitted.

Purification of protease and detergent forms of rabbit aminopeptidase N

The main steps of the purification procedure are summarized in Scheme I and Table I.

Common steps. Frozen mucosa (600 g) was cut into pieces and gently stirred for 30 min in 2400 ml of buffer A. Part of the mucus was removed by gauze filtration and a 15 min centrifugation at 3000 × *g*. A 3 h centrifugation at 30 000 × *g* of the 3000 × *g* supernatant yielded a second pellet which was suspended in 1 l of buffer A containing 3% Emulphogen. The suspension was stirred overnight at 4°C and centrifuged for 1 h at 30 000 × *g*. The resulting solution was brought up to 0.5 saturation ammonium sulfate and the precipitate containing the aminopeptidase activity was dissolved in 100 ml of buffer A containing 1% Emulphogen. The gel thus obtained was dialyzed against 5 l of buffer A. The resulting solution was cleared up by centrifugation and brought to 0.2 saturation ammonium sulfate. After a 30 min centrifugation at 30 000 × *g*, the upper gel phase was discarded and the lower phase was brought to 0.5 saturation ammonium sulfate. The new precipitate was dissolved in minimum volume of buffer A to give a solution (Crude Emulphogen extract) which was dialyzed against 5 l of buffer A. Beyond this point, the two forms were purified independently.

Purification of the protease form. The above solution made 0.1 M in Tris-HCl (pH 8.0) was incubated at 4°C for 2.5 h with trypsin (0.25 mg/ml). Hydrolysis was stopped by phenyl methylsulfonyl fluoride (1 mM). After dialysis against buffer A, the solution was filtered through a Sepharose 6-B column (3 cm × 400 cm) equilibrated with the same buffer. Fig. 1A shows that the aminopeptidase N activity emerged as a symmetrical peak at 2.15 void volumes. The fractions with specific activities higher than 6000 were pooled and dialyzed against buffer A containing 0.2 M KCl. The aminopeptidase A



Scheme I

Flow sheet of the purification of the protease (p-APN) and detergent (d-APN) forms of rabbit intestinal aminopeptidase N. Con-A, concanavalin A.

which emerged with the void volume of the column due probably to persisting aggregation was readily separated at this stage from aminopeptidase N.

The last purification step was a chromatography on a hydroxyapatite column (3 cm × 6 cm) equilibrated with a 10 mM monopotassium phosphate buffer (pH 6.0) containing 0.2 M KCl. The column was washed with the buffer and eluted as a symmetrical peak by a linear phosphate concentration gradient (2 × 450 ml) from 10 mM to 200 mM.

Purification of the detergent form of rabbit aminopeptidase N. For this purification, the trypsin digestion of the crude Emulphogen extract was omitted (Scheme I) and the detergent form was maintained in solution by 1% (v/v) Emulphogen in all buffers. The buffers also contained benzamidine (1 mM) and phenyl methylsulfonyl fluoride (1 mM) to keep to a minimum undesirable degradations. The crude Emulphogen extract was directly filtered through Sepharose 6-B in the same type of column as before. The elution profile is reproduced in Fig. 1B. The detergent form emerged at approx. 1.95 void volumes. It was again well separated from the detergent form of aminopeptidase A which emerged at 1.7 void volumes.

The pooled fractions containing the aminopeptidase N activity were loaded onto a 60 ml Concanavalin A-Indubiose column equilibrated with a 10 mM

TABLE I

RESULTS OF THE PURIFICATION OF THE PROTEASE AND DETERGENT FORMS OF AMINOPEPTIDASE N

Anti-I, anti impurities immunoabsorbant; Con A, concanavalin A.

Step	Yield (%)	Specific activity (units/mg protein)
Common steps		
Gauze filtration of homogenate	100	420
3000 × *g* supernatant	60	430
30 000 × *g* pellet	55	—
Emulphogen extract	45	400
Ammonium sulfate precipitation (0.5 saturation)	35	580
Ammonium sulfate precipitation (0.2—0.5 saturation)	25	1 350
Protease form		
Trypsin treatment	25	1 300
Sepharose 6-B filtration	23	7 000
Hydroxyapatite chromatography	16	15 000
Detergent form		
Sepharose 6-B filtration	23	9 500
Con A-Indubiose chromatography	16	—
Anti-I immunoabsorbant chromatography	12	—
DEAE-cellulose chromatography	10	15 000

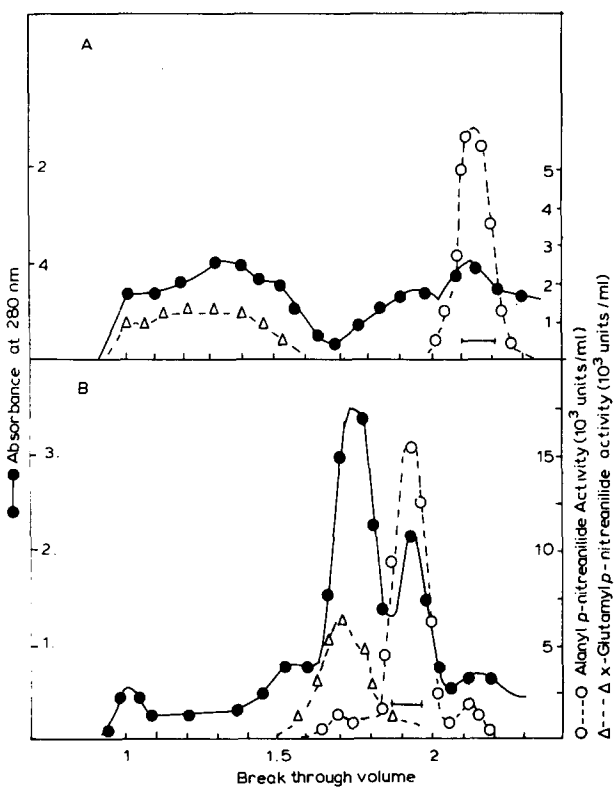


Fig. 1. Filtration through Sepharose 6-B: (A) Of the tryptic digest of the crude Emulphogen extract (purification of the protease form in Scheme I); no Emulphogen. (B) Of the same extract before trypsin treatment (purification of the detergent form in Scheme I); 1% Emulphogen.

Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Approximately 70% of the total activity migrated unretarded when the column was eluted with the same buffer.

Beyond this point, no conventional method could be found for increasing the specific activity of the detergent form above 12 000 compared to 15 000 for the most purified protease form. Antiaminopeptidase immunoabsorbant chromatography was unsuccessful because of heavy activity losses during elution and an entirely new technique must be worked out. The principle of this technique was to selectively precipitate aminopeptidase by the corresponding antibody, thus leaving the impurities in solution. In a second step, 'anti impurities' (anti I) antibodies were raised and then used for reverse immunoabsorbant chromatography under suitable conditions.

In practice, the pooled active fractions emerging from Sepharose 6-B were precipitated by goat antibodies directed against the protease form. Goat anti impurities antibodies were purified and coupled with Indubiose ACA 22 by the Avrameas technique [17]. Then, the detergent form emerging from Concanavalin A was dialyzed against a 10 mM phosphate buffer (pH 7.4) and introduced into a column filled with the above immunoabsorbant material and equilibrated with the phosphate buffer. Each unretarded active fraction was tested by rocked immunoelectrophoresis using anti impurities antibodies. The fractions judged pure were pooled and dialyzed against buffer A. The resulting solution was freed from traces of remaining concanavalin A and antibodies by passage through a short DEAE-cellulose column. This passage also permitted to concentrate the solution and to reduce the Emulphogen concentration to 0.1%. Table I shows that the detergent form finally obtained had the same specific activity as the most purified protease form.

Results

Characterization by crossed immunoelectrophoresis of the detergent and protease forms of aminopeptidase N

As reported earlier [5,8], the junction between the hydrophilic and the hydrophobic domains of brush border hydrolases is readily accessible to proteolytic attack. Fig. 2 shows that any uncontrolled conversion of the labile detergent forms into the more stable protease forms can be easily detected and quantified using the crossed immunoelectrophoresis technique associated or not with specific staining of enzymatic activities. This technique is very sensitive and convenient, especially in the case of the detergent form of aminopeptidase N.

Charge heterogeneity of the protease form

This heterogeneity was revealed by DEAE-cellulose chromatography and gel electrophoresis in the absence of sodium dodecyl sulfate.

As shown by Fig. 3, the protease form yielded a broad peak on DEAE-cellulose. It also yielded a broad band by gel electrophoresis (insert of Fig. 3, lane p). In contrast, narrow bands were obtained with aliquots taken from the ascending, top and descending parts of the DEAE-cellulose peak (fractions 32, 44 and 56) and these bands were situated at different positions on the gel

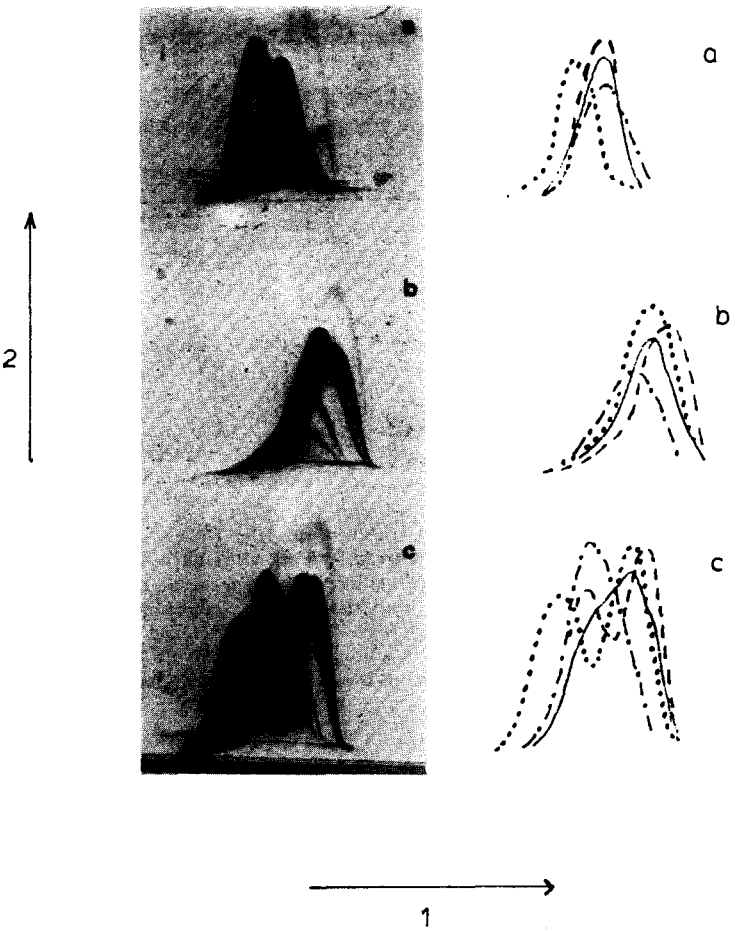


Fig. 2. Crossed immunoelectrophoresis of Emulphogen and papain solubilized rabbit brush border proteins. See text for experimental conditions. The gel used in the second dimension (arrow 2) contained 0.5 mg/ml of guinea pig immunoglobulins raised against the Emulphogen extract of rabbit intestinal brush border membrane. Applied samples contained 30 units of aminopeptidase N. (a) and (b), respectively, detergent and papain solubilized proteins; (c) mixture of equal amounts of (a) and (b). On the left, picture taken after protein staining by Coomassie Blue. On the right, reproduction of the immunoprecipitates of aminopeptidase N (· · · · · ·), aminopeptidase A (———), sucrase-isomaltase (- - - - - -) and maltase (· — · — ·) revealed by specific chromogenic substrates.

(lane 32, 44 and 56). A mixture of fractions 32 and 56 gave 2 well separated bands (lane 32 + 56). These data are consistent with the view that the protease form contains isoenzymes with different net charges. Fig. 4 indicates a total immunological homology between the isoenzymes. No contamination of the preparation could be detected by this technique.

Moreover, the above fractions 32 and 56 led to the same kinetic parameters towards 2 specific aminopeptidase substrates, Leucine *p*-nitroanilide (K_m, 0.65 M; k_{cat}, 66 s^{-1}) and alanine *p*-nitroanilide (K_m, 0.28 mM; k_{cat}, 38 s^{-1}). A similar charge microheterogeneity probably exists in the detergent form. But, it is more difficult to demonstrate because of the much slower electrophoretic migration of this form.

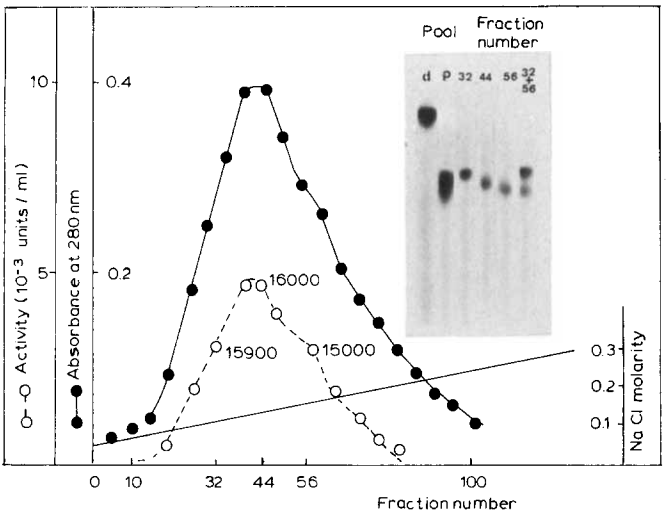


Fig. 3. DEAE-cellulose chromatography of the protease form of aminopeptidase. The enzyme preparation was dialyzed against buffer A containing 50 mM NaCl and charged on a DEAE-cellulose column (2 × 25 cm) equilibrated with the same buffer. The aminopeptidase was eluted by a linear NaCl concentration gradient (2 × 250 ml) from 0.05 M to 0.3 M. The specific activities of the fractions are indicated by figures along the peak. ———, NaCl concentration gradient. Insert: polyacrylamide gel electrophoresis of the pooled fractions emerging from the column (p), of fractions 32, 44, 56 of the DEAE-cellulose peak, and of a mixture of fractions 32 and 56. The migration of the detergent form of aminopeptidase is indicated for purpose of comparison.

Ultracentrifugation assays

The sedimentation coefficient of the protease form was found to be concentration dependent. Extrapolation to zero concentration led to a value $s = 7.4$ S in good agreement with that proposed by Takesue [11].

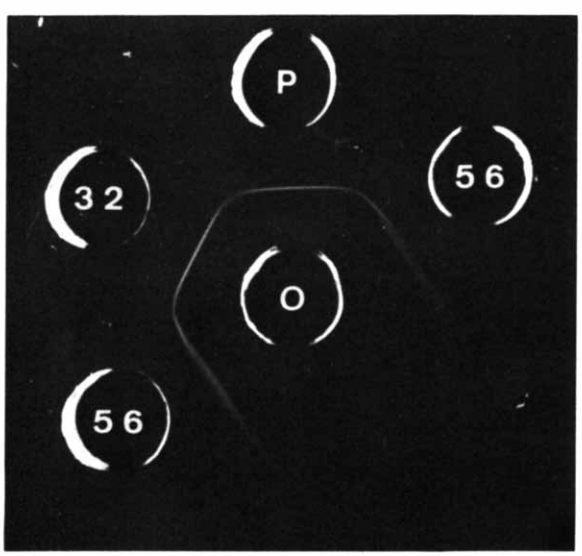


Fig. 4. Double immunodiffusion of the protease form eluted from the DEAE-cellulose column (Fig. 3). Aminopeptidase (6 μg) present in fractions 32, 56 and in the pooled active fractions (p) was diffused against 10 μl of goat serum directed against fraction (p) and deposited in well O.

By the equilibrium technique of Yphantis, a linear log $y = f(r^2)$ plot was obtained. Taking into account a partial specific volume of 0.682 ml/g, an apparent molecular weight of 120 000 ± 5000 could be calculated for the protease form. This value is similar to that already reported for the rabbit renal enzyme [18], but it is approximately twice as low as that found for pig intestinal aminopeptidase N under similar conditions.

The hydrophobic anchor

The hydrophobic peptide originating from the rabbit detergent form was purified by the technique already described in the case of the porcine enzyme [5]. After labeling with ^{125}I [19], the protein (3—5 mg per ml of a 100 mM Tris-HCl buffer (pH 7.8) containing 0.1% Emulphogen) was incubated at 4°C for 2.5 h with 0.25 mg/ml of Sepharose bound trypsin. Electrophoresis of the purified peptide in the presence of sodium dodecyl sulfate yielded a single radioactive band corresponding to an apparent molecular weight of 8500. This value is similar to that already reported for the porcine enzyme [5].

However, the hydrophobic peptide may be expected to bind more detergent molecules than the hydrophilic proteins serving for gel calibration [20] leading to overestimated molecular weight values. To test this possibility, an alternative and more reliable isotopic dilution method was worked out.

An exactly measured amount (b_0 cpm, x nmol) of the labeled peptide (peptide P_1) was mixed with an exactly measured amount (a_0 nmol) of unlabeled detergent form and the mixture was incubated with trypsin. Mild conditions were used for this step in order to avoid any degradation of the peptide. In return, the detergent form was not completely converted into the protease form and the amount actually generated (y nmol) was determined by chromatography on Sepharose 6-B as described in Fig. 5 or phenyl Sepharose. With this latter hydrophobic chromatography, the protease form was eluted unretarded with a 100% yield. Thus, the amount of this form could be determined with the desirable accuracy. The peptide P_2 also shown in Fig. 5 is a mixture of P_1 and of the unlabeled peptide yielded by the unlabeled detergent form, thus creating conditions for isotopic dilution. P_2 was purified in the usual manner. The situation at this point may be summarized as follows:

$$P_1 + \text{d-APN} \xrightarrow{\text{Trypsin}} P_2 + \text{p-APN} + \text{d-APN}$$

nmol:	x	a_0	$(x + y)$	y	$(a_0 - y)$
cpm:	b_0	0	b_0	0	0

In this scheme, d- and p-APN designate, respectively, the detergent and protease forms of aminopeptidase N.

The amino acid compositions of the peptides calculated for the same radioactivity (b_0 cpm) are indicated in Table II. The dilution factor ($(P_2/P_1) = x + y/x$) was derived from the average of the ratios for each amino acid. It served for the calculation of x, and this latter permitted to calculate the molecular formula of the peptide by the method of Delaage [21].

In a typical assay reported in Table II, the radioactivity b_0 of P_1 was $4.2 \cdot 10^6$ cpm; a_0 was equal to 50 nmol and y to 25 nmol (conversion yield, 50%). The average value of the dilution factor was 6.75 (x = 4.3 nmol). These param-

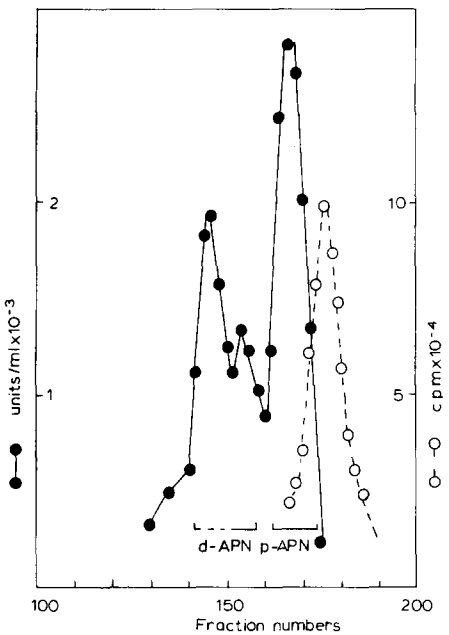


Fig. 5. Filtration through Sepharose 6-B after tryptic cleavage of a mixture of ^{125}I-labeled peptide P_1 and unlabeled detergent form. The column (1 × 300 cm) was equilibrated and eluted with buffer A containing 1% Emulphogen. Fraction volume, 1 ml. The detergent and protease forms were characterized by crossed immunoelectrophoresis. The emergence of the labeled peptide is indicated by the interrupted line. d-APN and p-APN, respectively, the detergent and protease forms of aminopeptidase N.

TABLE II

AMINO ACID COMPOSITION OF THE PEPTIDE AFTER 24 h HYDROLYSIS

	Residues per *b* cpm		Dilution factor (P_2/P_1)	Residues/mol	
	P1	P2		Calculated values	Next integer
Asp	10.6	74	7	2.9	3
Thr	5.45	29.8	5.4	1.5	1—2
Ser	11.0	81.3	7.4	3.0	3
Glu	15.0	101	6.7	4.1	4
Pro	3.6	22.7	6.3	0.99	1
Gly	2.1	15.0	7.1	5.8	6
Ala	12.9	89.7	6.9	3.5	3—4
Val	9.8	61	6.2	2.7	3
Ile	8.6	56	6.5	2.4	2
Leu	16.0	107	6.7	4.4	4
Tyr	4.4	30.4	6.9	1.2	1
Phe	4.7	34.6	7.4	1.3	1
His	2.3	16.2	7.1	0.6	1
Lys	7.5	50.5	6.7	2.0	2
Arg	3.3	22.9	6.9	0.9	1
Number of residues					36—38
Molecular weight					3710—3880

eters led to an apparent molecular weight of about 3800 (36—38 residues) for the detergent free peptide.

Detergent binding

The Sepharose 6-B filtration assays presented in Figs. 1B and 5 were carried out in the presence of 1% Emulphogen. Under these conditions, calibration of the column with the hydrophilic protease forms of rabbit and pig aminopeptidase N indicated for the rabbit detergent forms and for the peptide molecular weight values, of respectively, 200 000—220 000 and 70 000 ± 10 000. These values are considerably higher than those determined in the absence of detergent. The best assumption already put forward earlier to explain this discrepancy is that the hydrophobic domains of both detergent form and peptide bind a substantial amount of Emulphogen which, in the case of the peptide, may be estimated to 90 monomers per mol. It is of interest to note that, in the presence of 1% Emulphogen, the apparent molecular weight of the detergent form agrees well with the sum of the molecular weight of the protease form plus that of the peptide.

The molecular weight found for the peptide by gel electrophoresis in the presence of sodium dodecyl sulfate is intermediate (8500). This value may be due to the fact that sodium dodecyl sulfate is a 'hard' detergent able to combine with the reference hydrophilic proteins after previous denaturation, thus compensating a part of the error.

Monomeric structure of the detergent and protease forms

Both reduced-carboxymethylated forms were dissolved in a buffer containing 1% sodium dodecyl sulfate. Gel electrophoresis of the solution in the presence of 0.1% sodium dodecyl sulfate yielded a single narrow band corresponding to a molecular weight of 120 000. This value is identical to that obtained earlier for the protease form by ultracentrifugation in a non dissociating medium, showing that the protease form is monomeric after solubilization from the membrane. The same conclusion can be reached in the case of the detergent form whose molecular weight in the presence of Emulphogen (200 000—220 000) is fully accounted for by the weight of the denatured monomer (120 000) and that of the peptide-detergent complex (70 000).

N-terminal residues in proteins and peptides

All the protease forms obtained in the course of the present work had a N-terminal residue of serine. By contrast, an N-terminal tyrosine was identified in the detergent form and in the peptide.

Amino acid, sugar and Zn content of the two forms

The amino acid composition of the protease and detergent forms is given in Table III. The molecules are composed of approximately 1000 amino acid residues. Sugars amount to approx. 12—13% of the total (23% in the porcine enzyme). The sialic acid content was too low to be measured. Taking into account the relative imprecision of the results listed in Table III, no effort was made to calculate by difference the composition of the peptide and to compare it with that yielded in a more direct way by the isotopic dilution technique.

TABLE III

AMINO ACID COMPOSITION OF THE TWO FORMS OF AMINOPEPTIDASE

The amino acid composition was calculated by the method of Delaage [21] using 105 000 for the total weight of the amino acids. n.d., not determined.

	Residues/mol	
	Detergent form	Protease form
Asp	121	123
Thr *	66	70
Ser *	79	77
Glu	113	113
Pro	55	50
Gly	53	50
Ala	82	77
½ Cyst	n.d.	n.d.
Val	71	68
Met	16	13
Ile	54	47
Leu	117	110
Tyr	35	36
Phe	46	45
His	23	19
Lys	30	27
Arg	41	40
Trp **	23	23
Number of residues	1 025	988
Total weight of residues	114 789	110 895
Glucosamine	44	38
Galactosamine	15	14
Amino sugars (%)	8	7.4
Neutral sugars (%)	5.6	4.7
Total molecular weight	134 000	127 000

* After extrapolation to zero time of the results obtained after 24, 48 and 78 h hydrolysis.
** Colorimetric method of Spies and Chambers [22].

The total molecular weight calculated by summation of the amino acids and sugars is slightly higher than that determined earlier by ultracentrifugation and gel electrophoresis.

The protease form was found to contain one zinc atom per mol (two atoms in the case of dimeric aminopeptidases [1,9,10,23–26]). Complete inhibition by 1 mM EDTA at pH 6.0 may suggest that intestinal aminopeptidases are zinc enzymes. However, no inhibition was noted at pH 8.0 under the same conditions.

Discussion

The biosynthesis, intracellular processing and mode of integration of intrinsic membrane proteins are amongst the most challenging problems of cellular biology. The model selected for the last few years in our laboratory has been the hydrolases bound to the intestinal and renal brush border membrane, especially the aminopeptidases which are major and undoubtedly important constituents of this membrane. In the course of the present work, the detergent

and protease forms of rabbit intestinal aminopeptidase N have been prepared in a state of purity sufficient for chemical investigations. The preparations have also been used to raise antibodies designed for the specific labeling of the enzyme in situ by immunofluorescence as reported in a forthcoming paper. The specific activities of the detergent and protease forms were shown to be the same, suggesting that the presence of the hydrophobic anchor in the first did not affect its catalytic properties.

An interesting aspect of the work was to find that full purification of the detergent form required a special technique using an anti impurities immunoabsorbant. In this technique that we propose to call 'reverse immunoabsorbant chromatography', the antigen to be purified leaves the column unretarded with a high yield while the impurities are retained. It may be helpful in other cases when two fully cross reacting but chemically different forms of the same enzyme are available.

Although carefully purified, the protease form of aminopeptidase N and probably also the detergent form displayed charge microheterogeneity. Since the differently charged entities present in these preparations were indistinguishable by immunological and enzymatic methods, this microheterogeneity was assumed to be due, as it is often the case with glycoproteins, to a variability of the sugar moiety. Sialic acid being absent, the variability must be attributed to the amino sugars as it is the case for the rat aminopeptidase [9].

Moreover, the detergent form was found to give rise to several protease forms of different size. This findings is not surprising since limited proteolysis like that occurring at the hydrophilic-hydrophobic junction of the detergent form are rarely quite specific. Nevertheless, all protease forms so far characterized possessed the same N-terminal residue, suggesting that a major splitting occurs at the above mentioned junction and that the observed size differences are due to additional cleavages at the C-terminal end of the molecules. More detailed information about the variability of the protease form will be given in a forthcoming paper.

It was noteworthy that the most purified protease form obtained in the present work will contained a strongly antigenic contaminant leading to unspecific labeling of ultrathin frozen jejunum section by immunofluorescence (unpublished results). This contaminant is probably present in all preparations of bursh border hydrolases obtained in our and other laboratories and may be expected to contract very strong interactions with the enzymes.

A further remark concerning the purification was that the detergent form was easily converted into the protease form by endogeneous proteolytic enzymes at the beginning of the procedure. The detergent form was more stable after the Sepharose 6-B filtration by which the majority of these enzymes are probably removed.

The general features of the structure of rabbit aminopeptidase N are very similar to those already reported for the porcine enzyme [1,5]. In particular, the molecule was shown to be composed of a hydrophilic domain solubilized by proteolytic digestion of the membrane and of a hydrophobic anchor. However, a major difference was that the detergent and protease forms of the rabbit enzyme were found to be monomeric after solubilization from the membrane. With the exception of aminopeptidase N from rabbit kidney [18], the other

intestinal and renal aminopeptidases characterized so far are dimeric. This monomeric structure facilitated the chemical identification of the anchor. It also demonstrated that a monomeric enzyme in an aqueous environment can display full aminopeptidase activity. But, it does not exclude the possibility that at least some of the properties of aminopeptidases in the membrane require a dimeric or even a polymeric structure.

The apparent molecular weight of the peptide detached from the detergent form during its conversion into the protease form was observed to be considerably affected by the presence of detergents. A new isotopic dilution method permitted to show that this molecular weight is not 8500 as reported for the peptide of the porcine enzyme, but only 3800, the rest representing probably bound detergent molecules. The molecular weight of peptides from other brush border hydrolases may have also been overestimated. An important consequence of this finding is that the real anchor, which should be still shorter than the peptide and therefore should contain less than 36—38 residues, suffices for the binding at the membrane surface of a 'head' containing more than 1000 amino acid residues and also sugars. The real size of the anchor must be taken into account in the investigations concerning the tridimensional structure and topology of this anchor inside the membrane.

The amino acid composition of the peptide could be accurately ascertained because of the monomeric structure of the corresponding enzyme (Table II). It shows that the peptide is predominantly, but not exclusively, hydrophobic. It is not yet known if the hydrophilic residues of the peptide are really included in the anchor plunging into the membrane core or if they at least partly originate from the region of the 'head' just protruding from the membrane surface.

Finally, a comparative determination of N-terminal residues in both monomeric enzyme forms and in the peptide clearly confirmed that the peptide and consequently the anchor were located at the N-terminal of the aminopeptidase chain. This view, expressed for the first time for pig intestinal aminopeptidase N [5] has recently been extended to other brush border enzymes using N-terminal residue determination [27]. It has also been reinforced by the identification of a number of hydrophobic residues in the N-terminal region of the detergent form of rabbit intestinal isomaltase [28] and renal dipeptidyl peptidase IV [27]. This insertion of brush border hydrolases by the N-terminal of the chain requires a modification of the scheme originally proposed by Blobel et al. [29] for secretory proteins and also for several membrane proteins inserted by their C-terminal.

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