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TOPOLOGICAL STUDIES ON THE HYDROLASES BOUND TO THE INTESTINAL BRUSH BORDER MEMBRANE

II. INTERACTIONS OF FREE AND BOUND AMINOPEPTIDASE WITH A SPECIFIC ANTIBODY*

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Summary

The position of the intestinal brush border aminopeptidase with respect to the lipid bilayer has been investigated with the aid of right side out vesicles prepared from the brush border and an immunological technique using an unlabelled or peroxidase-labelled antibody specific for aminopeptidase. The finding that the bound form of the enzyme was almost as readily inhibited and agglutinated as the free form during incubation with the antibody was consistent with the view that the majority of the aminopeptidase surface emerged from the bilayer. This finding was entirely corroborated by the observation that only a few antigenic determinants were not free to react with the antibody in bound aminopeptidase.

This immunological technique may be applied to other membrane proteins provided that preparations of the pure proteins and of specific antibodies are available.

Introduction

Several findings reported by other workers [1—3] and in the first paper of this series [4] have strongly suggested that all known brush border hydrolases are attached to the external side of the membrane, i.e. the side directed towards the lumen. Moreover, the ready solubilization of some of these enzymes by papain digestion of closed, right side out vesicles originating from the brush border was consistent with the view that a large part of these molecules emerged from the plane of the lipid bilayer [4].

*This work is a part of the Dr. Sc. Thesis of D. Louvard, Faculty of Sciences, Marseilles.

The purpose of the present work was to explore the potentialities of an immunochemical technique for obtaining a more precise insight into the topography of aminopeptidase (EC 3.4.11.2) which is known to be one of the major protein constituents of the brush border membrane [5]. The above expressed views were fully supported by the finding that the bound enzyme interacted with a specific antibody almost as readily as the free form.

Materials and Methods

Materials

Horse radish peroxidase (EC 1.11.1.7) Grade I (RZ = 3) was purchased from Böhringer. Papain (EC 3.4.22.2), an NBC product, was activated prior to use by an already described technique [6]. Sodium dodecyl sulfate was obtained from Merck, Agarose from Touzart et Matignon and Indubiose AcA-3/4 (a Biogel P 300 substitute with better flow properties) from Industrie Biologique Française. Glutaraldehyde was a TAAB Laboratory product, as recommended by Avrameas et al. [7]. The protein MOPC 173 [25] and the antiserum of rabbits immunized with Balb/c serum [25] from which the MOPC 173 antibody was purified were kind gifts of Professor Michel Fougereau. All other products were reagent grade.

Enzymes

"Free" (or molecularly dispersed) aminopeptidase was purified to homogeneity by a technique [5] including solubilization from pig intestinal mucosa by Triton X-100, treatment of the extracts by trypsin and chromatography on DEAE-cellulose at pH 8.0 and Sephadex G 200. The purity of the preparations was checked by polyacrylamide electrophoresis at pH 8.1 and ultracentrifugation. The membrane-bound aminopeptidase was included in the limiting membrane of closed, right side out vesicles [4] prepared by gentle homogenization of pig jejunal mucosa in the absence of EDTA followed by several low and high speed centrifugations in sucrose solutions of adequate densities [8]. Some of these centrifugations were performed in the presence of Ca^{2+} in order to remove the rough microsomes from the vesicles [8, 9].

The material designated "Papain supernatant" was a mixture of the hydrolases (aminopeptidase, alkaline phosphatase, several disaccharidases, γ-glutamyltransferase) and other membrane surface constituants solubilized from the above vesicles by papain treatment [4].

Determination of enzyme activities

Aminopeptidase, alkaline phosphatase and maltase activities were measured as described earlier [8, 4] with the aid of L-alanine *p*-nitroanilide (Cyclo Chemical Co.), *p*-nitrophenylphosphate (Sigma) and maltose (Merck), respectively. Peroxidase was evaluated colorimetrically [10] using *o*-dianisidine (Sigma) as the substrate. Specific activities and molar concentrations were computed from the following data: Extinction coefficient ($E^{1\,\%}_{1\,cm}$) at 280 nm: aminopeptidase, 15.6; aminopeptidase antibody, 13.5; Fab fragment, 13.0; peroxidase, 7.3 (21.9 at 403 nm). Molecular weight: aminopeptidase, 280 000; antibody, 150 000 and peroxidase, 40 000.

Obtention of an antibody specific for aminopeptidase

Rabbit immunization and collection of antisera were described in a previous publication [4]. As a rule, each animal received a total of 6 mg of pure aminopeptidase (1 mg subcutaneously, 1 mg intramuscularly and 2 x 2 mg intraveinously). The antibody was purified to homogeneity in a single step by affinity chromatography. For this purpose, aminopeptidase (34 mg) was bound with a 75% yield to an Indubiose suspension (30 ml) activated by glutaraldehyde as described by Ternynck and Avrameas [11]. The resulting suspension was poured into glass tubes (3 cm inner diameter) to form 6-cm high columns which were charged with the aminopeptidase antiserum. The unretarded proteins were washed with phosphate-buffered saline (150 mM NaCl/10 mM K_2HPO_4 adjusted to pH 7.2 with H_3PO_4) and the antibody was eluted later by passage of a 0.2 M glycine·HCl buffer at pH 2.2. The eluate was neutralized with 1 M K_2HPO_4, dialyzed against the phosphate-buffered saline, concentrated to 5 mg/ml by vacuum dialysis, filtered through 0.22 μM Millipore plates and divided into 0.5 ml fractions which were kept separately in sealed glass tubes at —80°C until use. The preparations heated at 60°C during 15 min in the presence of 1% sodium dodecyl sulfate were observed to give a single band by electrophoresis in a 7.5% polyacrylamide gel at pH 7.2 in the presence of 0.1% sodium dodecyl sulfate. It also yielded a single symmetrical peak by ultracentrifugation in the phosphate-buffered saline. The corresponding sedimentation coefficient ($s_{20,w}$) was found to be 6.7 S, a value in good agreement with those commonly observed for γ-globulins.

Preparation of the Fab fragment

Some inhibition assays reported below were performed with the aid of the fragment designated Fab fragment resulting from a limited proteolysis of the antibody with papain. After a 16 h incubation at 37°C in the conditions described by Porter [12], the hydrolysate was freed from inactive degradation products, mainly Fc, by passage through the Indubiose-aminopeptidase column already used before the purification of the antibody. The eluate by the glycine·HCl buffer was neutralized, dialyzed against the phosphate-buffered saline, vacuum concentrated, filtered through 0.22 μm Millipore plates and charged into a Sephadex G 100 column (200 cm x 1 cm) equilibrated with a 0.1 M potassium phosphate buffer at pH 6.8. The fractions under the third peak emerging from the column at 1.65 breakthrough volume (molecular weight, about 50 000; 85% of the total proteins emerging from the column) were pooled, dialyzed against the phosphate-buffered saline, vacuum concentrated and filtered through 0.22 μm Millipore. The final product (4 mg of Fab from 25 mg of antibody) was found to be homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by analytical ultracentrifugation under the same conditions as above (sedimentation coefficient $s_{20,w}$, 3.5 S).

The specificity of the antibody preparations for aminopeptidase was checked by immunoelectrophoresis against a papain supernatant [4]. The single visible precipitin line could unambiguously be identified as that of aminopeptidase by its position on the plate and its activity against the chromogenic substrates of the enzyme [4]. Besides, isolated pig enterocytes were prepared

from the jejunal mucosa by the method of Kimmich [13] and an aliquot of the resulting suspension (1 ml; 15 mg of proteins per ml) was incubated for 2 h at 25°C with 100 µl of a 0.3 mg/ml solution of the peroxidase-labelled antibody (see later). After 3 washings with the phosphate-buffered saline at 4°C, the cells were resuspended in the initial volume of phosphate-buffered saline and a drop of the suspension was mixed with a drop of the *o*-dianisidine solution serving for peroxidase determination. In a light microscope (magnification x 1000), the brush border region of the plasma membrane appeared deeply colored in brown whereas no detectable staining could be observed in the baso-lateral region. The leucocytes contaminating the pig enterocyte preparations were not stained either. The same remark applied for rat and guinea pig enterocytes. Finally, no cross-reactions could be discerned between the pig aminopeptidase antibody and rat aminopeptidase.

Preparation of the antibody-peroxidase conjugate

The coupling of the two proteins was realized under the conditions described by Avrameas et al. [7]. As shown by Fig. 1, passage of the resulting mixture through Indubiose yielded 3 well separated peaks absorbing at 403 nm, a wavelength characteristic for peroxidase. The first emerged in the position expected for proteins with a molecular weight of about 200 000 and, therefore, it could be assumed to contain the conjugate of 1 mol each of peroxidase and antibody (molecular weight 190 000). The position of this conjugate in the first peak was more precisely ascertained by measuring in each fraction the absorbance ratio at 403 and 280 nm. Fractions with ratios between 0.380 and 0.450 (value for the pure 1:1 conjugate, 0.390) were pooled as indicated in Fig. 1. The mixture was concentrated to 3 ml by vacuum dialysis (0.273 mg of

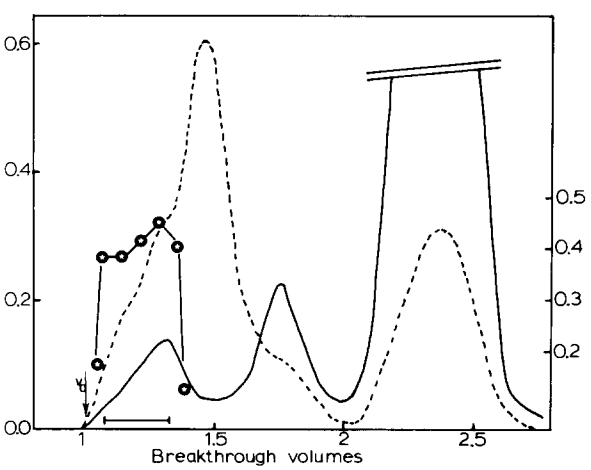

Fig. 1. Obtention of the 1:1 antibody-peroxidase conjugate. The glutaraldehyde-activated peroxidase (10 mg in 1 ml) was incubated for 24 h at room temperature with the pure antibody (5 mg in 1 ml) and 0.2 ml of a 0.5 M sodium carbonate/bicarbonate buffer at pH 9.5. The mixture was filtered through an Indubiose (AcA-3/4) column (200 cm x 1 cm) equilibrated and eluted by a 0.1 M potassium phosphate buffer at pH 6.8. The column was calibrated with bovine serum albumin, ovalbumin, and bovine chymotrypsinogen (molecular weights 69 000, 43 000 and 25 000, respectively). Fraction volume, 1 ml. Breakthrough volume, 53 ml. Flow rate, 6 ml/h. ●, 403/280, ratio of the absorbance of the fractions at 403 and 280 nm. Pooled fractions are indicated by an horizontal bar. - - - and —, $A_{403\ nm}$ and $A_{280\ nm}$, respectively.

proteins per ml; ratio, 0.400) and divided into 100 μl aliquots which were kept at −20°C until use. The peroxidase specific activity of the conjugate calculated from the absorbance at 403 nm was similar to that of the native enzyme and its ability to induce a 50% inhibition of aminopeptidase was at least 95% of that of the original antibody. The coupling of the anti-MOPC 173 antibody with peroxidase was carried out under the same conditions and yielded similar results.

Results

Inhibition assays

The fact that the membrane-bound aminopeptidase was able to interact with the antibody was first proved by the inhibition assays reported in Fig. 2. For these assays, a fixed number of enzymatic units present either in a solution of free aminopeptidase or in an equivalent volume of a suspension of vesicles were incubated with varying amounts of the antibody and the remaining activities were measured in each case. The upper diagram in Fig. 2 shows that the inhibition of the free enzyme readily proceeds up to about 65% and almost reaches completion for higher antibody-antigen molar ratio values. A 50% inhibition is attained for a 3-fold molar excess of the antibody and a 6-fold excess of the Fab fragment.

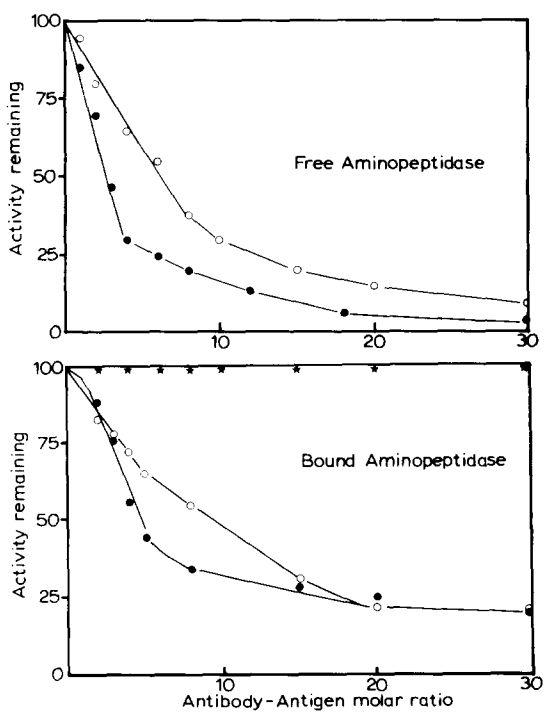

Fig. 2. Inhibition of aminopeptidase by the antibody (●—●) and Fab fragment (○—○). Upper diagram: the free enzyme (24 pmol) was mixed with 0—770 pmol of antibody or Fab fragment. The pH was adjusted to 7.3 by addition of the phosphate-buffered saline in a total volume of 75 μl. The mixtures were incubated for 2 h at 37°C and for 16 h at 4°C. The remaining aminopeptidase activity was measured on 20-μl aliquots. Lower diagram: same assays with vesicles. The symbol ★ designates the remaining alkaline phosphatase and maltase activities after incubation with the aminopeptidase antibody.

A similar series of assays performed with the bound antigen are depicted by the lower diagram of Fig. 2. The bound form is seen to be also inhibited by the antibody and the Fab fragment. The general aspect of the inhibition curves are the same as with the free form. However, a higher antibody concentration is necessary in this case to attain a 50% inhibition (5 molecules of antibody and 10 molecules of the Fab fragment per molecule of antigen, instead of 3 and 6),and a somewhat larger proportion of aminopeptidase activity (20% instead of 5—10%) is not inhibited even in the presence of a substantial excess of antibody. A further interesting aspect of the present assays was that other enzymatic activities included in the vesicles (alkaline phosphatase and maltase) were not affected during the incubation with the aminopeptidase antibody.

Precipitation and agglutination assays

Another experimental approach to the same problem was to compare the precipitation of molecules and the agglutination of vesicles resulting from incubation with the aminopeptidase antibody. In both cases, varying amounts of the antigen were added to a fixed quantity of the antibody. After incubation, the aminopeptidase inhibition effect reported above was measured and the insolubilized material was separated by a 3 min centrifugation in an IEC bench centrifuge operated at full speed (about 600 x *g*). It was checked that native vesicles were not sedimented in these conditions. Then, the proportions of the aminopeptidase activity present in the sediments were computed from those remaining in the supernatants due account being taken of the inhibition effect reported above. Fig. 3 shows that free aminopeptidase was readily and almost completely precipitated under these conditions whereas vesicles were agglutinated. But, in agreement with what was observed during the inhibition as-

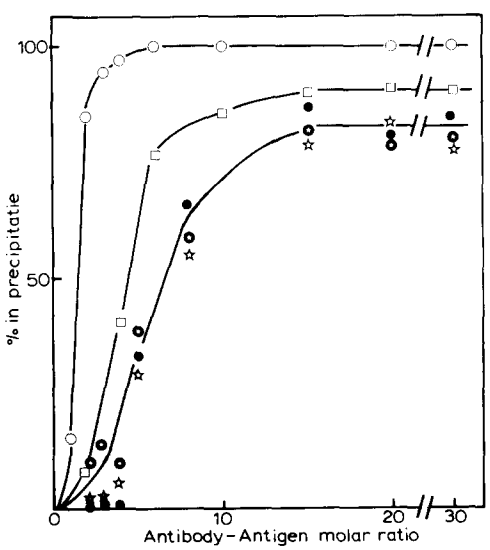

Fig. 3. Precipitation of free aminopeptidase and of aminopeptidase-containing vesicles. The antibody (616 pmol) was incubated for 2 h at 37°C and 16 h at 4°C with 22—308 pmol of antigen in a total volume of 150 µl of the phosphate-buffered saline. ○ and ●, free and bound aminopeptidase, respectively, ☆ and ✪, alkaline phosphatase and maltase. □, % of antibody bound to the agglutinated vesicles (see text).

says, higher antibody concentrations were required in this latter case and the maximal attainable degree of agglutination (80%) was significantly lower than that observed with free molecules. The cosedimentation of 3 activities included in the vesicles, namely aminopeptidase, alkaline phosphatase and maltase, under the influence of the antibody specific for aminopeptidase was also noteworthy.

Other agglutination assays were performed with the peroxidase-labelled antibody prepared as indicated in the Methods Section. The incubated mixtures tures were centrifuged first at 600 x *g* for 3 min and then at 15 000 x *g* for 15 min in order to spin down successively the agglutinated and total vesicles. The peroxidase activity found in the supernatants served for the calculation of the antibody amounts bound to the two above fractions in each assay. The ratios of these values are seen in Fig. 3 to be low when the antibody/antigen proportions were also low and then to increase to almost 100% in the presence of a large antibody excess (20—30 molecules per antigen molecule).

Vesicle agglutination was definitely slower than that of molecules. Equilibrium was already attained after 2 h at 37°C in the latter case whereas an additional incubation at 4°C for 16 h was required in the first.

Saturation assays

In a third series of assays, attempts were made to compare the maximum number of antibody molecules susceptible to be fixed by free and bound aminopeptidase. For this purpose, constant amounts of peroxidase-labelled antibody were incubated with varying quantities of both forms of the antigen and the resulting mixtures were centrifuged at 18 000 x *g* for 15 min. These centrifugations could obviously be expected to sediment the totality of the antibody attached to the vesicles. According to Fig. 3, the same situation was met when free aminopeptidase was employed. Hence, the supernatant peroxidase determinations could in all circumstances be assumed to yield by dif-

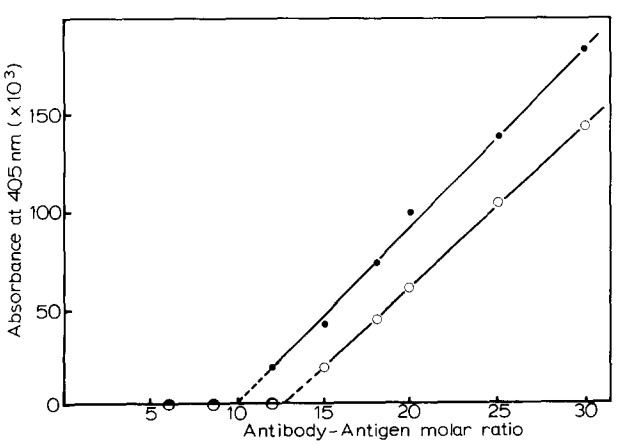

Fig. 4. Total number of antibody molecules fixed to free or bound aminopeptidase. The labelled antibody (616 pmol) was incubated as above in a final volume of 75 µl of the phosphate-buffered saline with varying amounts of aminopeptidase or vesicles (20.6—77.2 pmol). The measured absorbance at 403 nm indicated the antibody remaining in the 18 000 x *g* supernatants. ○ and ●, assays realized with aminopeptidase molecules or with vesicles, respectively. Each point is the average of 4 determinations.

ference the number of antibody molecules attached per antigen molecule.

Fig. 4 shows that no traces of antibody were found in the supernatants as long as the antibody/antigen molar ratio was lower than an apparently critical value. Above this value, detectable amounts were observed to appear and then to increase linearly. A point of great interest discussed in detail later was that the critical value obtained by extrapolation of the linear portions of the curves appears to differ significantly according to whether the free or bound antigen form was employed. In four determinations using the free form, the values obtained were 13, 13, 13 and 12 antibody molecules per antigen at the saturation. With the bound forms, the results were 10, 10, 10 and 11. Similar assays were carried out with the peroxidase-labelled heterologous anti-MOPC antibody. In this case, incubation with free or bound aminopeptidase induced no significant decrease of the peroxidase level in the supernatants, showing complete lack of unspecific binding in our assays.

Discussion

The position occupied by membrane proteins with respect to each other and to the lipid bilayer is an important factor controlling their function. The sealed, right side out vesicles recently obtained from duodenal and jejunal brush border [8] can be expected to be a suitable material for investigating the position of the hydrolases bound to this type of membrane.

The intestinal hydrolases such as aminopeptidase and the disaccharidases are known to be involved in the last degradations converting dietary proteins and polysaccharides into amino acids and glucose. Therefore, they can be assumed to be on the external side of the membrane, i.e. the side directed towards the lumen. This assumption was recently supported by the finding [4] reported in the Introduction that the hydrolases are readily detached from the vesicles by papain action. In addition, the observation that the forms detached by papain and Triton X-100 are different led to the hypothesis that the enzyme molecules are retained at the membrane surface by hydrophobic "anchors" interacting with the lipids of the bilayer [4].

The position of membrane proteins may also be related to their accessibility towards various reagents [14–16]. As rightly pointed out by Phillips and Morrison [17], the reagents employed for this purpose should not penetrate the lipid bilayer and be as specific as possible for certain surface groups of the proteins. The principle of their technique applied to intact human erythrocytes [17] is to iodinate the proteins by the system NaI-lactoperoxidase. Although probably more reliable than many others, this technique can be criticized on the following grounds: (a) iodine may be suspected to penetrate the lipid phase even in the presence of lactoperoxidase; (b) the iodination of proteins is a complex reaction involving several groups (tyrosine, histidine, etc . . .) which are often buried in the interior of the molecules and can in no case be considered as evenly distributed over the surface. At first sight, an immunological technique appears more promising because of the high specificity of the antigen-antibody interactions, the bulkiness of the antibody which should prevent any unwanted diffusion into the lipid bilayer and the known existence of multiple antigenic sites on the antigen surface [18].

An antibody preparation specific for intestinal aminopeptidase was shown to inhibit the free and bound forms of the enzyme. Both were also insolubilized during incubation with the antibody. Hence, although the bound enzyme required definitely more antibody to be inhibited and precipitated, and although the above processes were slower and less complete with this form, the antibody-antigen interactions did not appear to be fundamentally perturbed by the inclusion of the latter in the membrane structure limiting the brush border vesicles.

A more specific comment concerning Fig. 2 was that a 50% inhibition of both free and bound aminopeptidase required on a molar basis twice as much Fab fragment as intact antibody. This finding agrees with the results reviewed by Kabat [18] showing that, in the presence of an excess of free antigen, the two sites of the intact antibody participate in the binding. In the same connection, it is noteworthy that the second site of the antibody is also functional in the case of the membrane-bound antigen. This double interaction is possible on steric grounds*.

In addition, it was of interest that the fixation of the antibody to bound aminopeptidase did not induce any perceptible inhibition of alkaline phosphatase and maltase known to be included in the same vesicles (see later). Therefore, the fixation of a number of bulky antibody molecules around the corresponding membrane antigen does not appear to restrict the access of small substrates to the other enzymes.

A straightforward interpretation of the insolubilization assays described in in the Results section is not easy, because the mechanism by which free molecules precipitate [20] and vesicles agglutinate under the influence of an antibody are different [21, 22]. Fig. 3 shows that the formation of the tridimensional network responsible for free aminopeptidase insolubilization is induced by a very small antibody concentration increase. By contrast, vesicle agglutination is a more progressive and also slower process as if the initial antibody fixation was followed in this case by a reorganization of the protein pattern in the membrane. Alternatively, the slowness of agglutination of the vesicles may be due to their large size which lowers the frequency of productive collisions with the second arm of the antibody molecules. In the same order of idea, the peroxidase curve of Fig. 3 shows that vesicles agglutination does not occur before several antibody molecules are bound.

Another finding made in the course of the agglutination assays was that three enzymatic activities included in the vesicles, aminopeptidase, alkaline phosphatase and maltase, co-precipitated under the influence of the antibody specific for aminopeptidase. This probably means that the distribution of the enzymes over the membrane is not grossly heterogeneous. Otherwise, several vesicle populations with different compositions would be expected to result from homogeneization.

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*The distance between the two antibody sites in the T form is known to be 140 Å [19] whereas the average distance separating the centers of two bound aminopeptidase molecules can be estimated to be 160 Å if the following parameters are used: aminopeptidase is a globular protein (diameter, 86 Å) which represents 8% of the total proteins of the vesicles [5] and forms a regular lattice at the surface. The vesicles have an average diameter of 0.1 μm [8] and their volume is 1.5 μl per mg of protein (Louvard, D. and Maroux, S., unpublished experiments).

The curves presented in Fig. 4 illustrating the saturation of the free and bound antigen by the specific antibody will now be discussed. The linear portions of the curves correspond to high antibody concentrations including complete precipitation of the antigen. These conditions are known [18] to induce the antibody fixation by only one site. Consequently, the number of attached antibody molecules at the saturation can be expected to be equal to the number (n) of the antigenic determinants able to react simultaneously with the antibody. The latter may be assumed to be proportional to the available antigen surface and to correspond to complete occupancy. It was of great interest to find that this view was fully supported in the case of free aminopeptidase by the two following observations: (a) the portion of surface covered by a Fab or γ-globulin molecule at the moment of their fixation at an antigenic site has been estimated by X-ray crystallography [23] to be 50 Å x 40 Å = 2000 Å^2. The surface covered by 12—13 molecules (24 000—26 000 Å^2) agrees very well with the presumed surface (24 000 Å^2) of aminopeptidase, a globular protein with an approximate diameter of 86 Å [5]. (b) The parameter n, which is known to increase with the molecular weight of the antigen [18], can, as a working hypothesis, be assumed to be proportional to the surface. In this case, a linear relationship should exist between log M_r and log n. As indicated by Fig. 5, the points representing log M_r as a function of log n for five known proteins [18] actually fall along a straightline which also includes the point re-

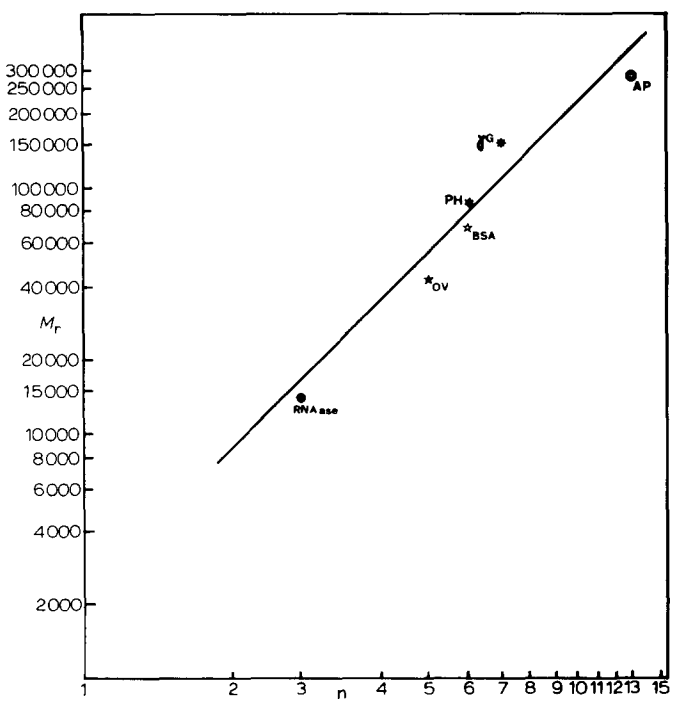

Fig. 5. Linear relationship between log M_r (molecular weight) and log n (number of antigenic determinants able to react simultaneously with the antibody) for several globular proteins [18] including: RNAase, pancreatic ribonuclease; OV, ovalbumin; BSA, bovine serum albumin; γG, human γ-globulins; PH, alkaline phosphatase of *Escherichia coli* (Lazdunski, C. et al., manuscript in preparation); AP, porcine intestinal aminopeptidase (this work).

lated to aminopeptidase assuming M_r = 280 000 and n = 13 for this protein. Hence, the value n = 12--13 indicated for free aminopeptidase by the corresponding saturation curve in Fig. 5 is very likely.

The above comments made in the case of free aminopeptidase confers more weight to the experimental finding that n = 10—11 with the bound form of aminopeptidase. This apparently significant difference is consistent with the view that a small portion only of the enzyme surface is either buried in the bilayer or masked by the anchor. An external position has also been suggested for the brush border maltase-isomaltase complex by electron microscopy after fixation of a ferritin-labelled antibody [24].

Against this conclusion, however, several objections may be formulated: (a) rearrangement of the protein and/or the lipid pattern in the brush border membrane during homogeneization and subsequent processing of the membrane. If true, the results obtained with vesicles would not be directly applicable to the native brush border. (b) the differences observed in Fig. 4 are not due to the existence of a masked portion in each enzyme molecule, but to the presence of non-reactive vesicles in the preparations. This objection is not supported by the fact that the totality of the aminopeptidase and disaccharidases bound to the vesicles is released by papain [4], and that, consequently all vesicles are right side out. Moreover, assays in progress in the Laboratory (Louvard, D. and Maroux, S., unpublished work) suggest that well defined determinants are masked in bound aminopeptidase. (c) The molarity of aminopeptidase in vesicles is underestimated due to the fact that the bound enzyme is less active than the free form. This possibility is unlikely for no detectable activity increase has ever been noted during enzyme solubilization by papain and Triton. (d) The position occupied by the bound enzymes are undetermined for the enzymes are free to move in a fluid environment. This idea is not supported by the finding that identical saturation curves are obtained after incubations at 37°C or 0°C.

Similar immunological techniques can probably be applied with equal success to other membrane proteins provided that pure samples of the protein and a labelled specific antibody are available. The label may be peroxidase as in the present case or any other easily detectable ligand such as radioactive iodine.

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