of synaptosomes to PGE\(_1\) would be consistent with the presence of a PGE\(_1\)-sensitive adenylate cyclase in pre-ganglionic fibres.

In our opinion the data presented here, as well as other unpublished work (V. Tomasi, C. Biondi, A. Trevisani, M. Martini & V. Perri), fit into the working hypothesis diagrammatically presented in Fig. 2. The stimulation of pre-ganglionic cholinergic fibres involves the activation of muscarinic receptors located on the membrane of interneurons containing dopamine and/or PGE\(_1\). The PGE\(_1\) released from the interneurons regulates ganglionic transmission by acting at two separated sites: (i) in the ganglion neurons as a modulator of the dopamine receptor (dopamine-sensitive adenylate cyclase); (ii) in the pre-synaptic nerve terminals by stimulating a PGE\(_1\)-sensitive adenylate cyclase.

The most speculative point of this scheme is the possible existence of interneurons capable of synthesizing and releasing prostaglandins, which in turn would act on different target cells. In this connexion, it may be pointed out that evidence has been given that in the liver PGE\(_1\) interacts with receptors located on the outer side of the hepatocyte membrane, activating a specific adenylate cyclase (Tomasi & Ferretti, 1975; Tomasi, 1976). It has been shown in addition that the liver cellular population responsive to PGE\(_1\) is different from the one metabolizing prostaglandins (Ferretti et al., 1976).


Structure–Function Relationship of Intestinal and Renal Brush-Border Membrane-Bound Aminopeptidases and Maltases

SUZANNE MAROUX, DANIEL LOUVARD, CHRISTIAN VANNIER and MICHEL SÉMÉRIVA

C.N.R.S., Centre de Biochimie et de Biologie Moléculaire, 31 Chemin Joseph-Aiguier, 13274 Marseille Cedex 2, France

The membranes of the intestinal and renal brush borders are highly specialized in a typical function of plasma membranes: the active transport of a number of metabolites. These membranes are known to contain a variety of hydrolases (aminopeptidase, disaccharidases, alkaline phosphatase and \(\gamma\)-glutamyltransferase). In the case of gut, these hydrolases are involved in the last step of intraluminal digestion. It has been postulated (Ugolev, 1972; Maroux et al., 1973; Malathi et al., 1973) that they also play a role in the transport of digested products across the membrane. The determination of the position occupied by these enzymes with respect to the lipid bilayer is of fundamental importance for a better understanding of their function.

Vol. 5
The brush-border membrane spontaneously forms right-side-out vesicles, which are very useful for studying the mode of integration of the surface proteins. An improved technique has been devised for the purification of large quantities of these vesicles (Louvard et al., 1973). We have first studied the relationship between the structure of the hydrolases and their mode of integration into the lipid bilayer.

Amphipathic structure of the brush-border hydrolases

The hydrolases are identified with surface glycoproteins of the membrane vesicles by immunoelectrophoresis techniques (Louvard et al., 1975a). They were solubilized either by papain treatment or by neutral detergent, without loss of activity. The enzymes released by these two different treatments have different molecular properties; in particular, the detergent-solubilized form strongly aggregates in the absence of detergent, whereas the papain-treated form remains in a monomeric state under the same conditions. They display different electrophoretic migrations on polyacrylamide gels; the detergent-solubilized form always migrates more slowly than the papain-treated form. The transformation of the detergent-solubilized form into the papain-solubilized form is possible either by papain treatment or also by trypsin treatment in the case of aminopeptidase and maltases.

These first results strongly suggest that, like other membrane-surface proteins, the brush-border hydrolases are amphipathic molecules. In other words, they are composed of a hydrophilic part bearing the hydrolytic activity and a hydrophobic part ensuring fixation to the lipid matrix. Detergents liberate the entire molecule, whereas papain liberates the hydrophilic part only by cleaving a strategically located peptide bond. Trypsin and papain hydrolyse the same peptide bond on the detergent-solubilized form and split the molecule into its two parts. Indeed, from a trypsin hydrolysate of the purified detergent-solubilized forms of intestinal and renal aminopeptidases and of intestinal maltases, the two parts of these molecules have been purified and characterized (Maroux et al., 1973; Maroux & Louvard, 1976; Vannier et al., 1976).

The hydrophilic parts contain sugar and have a high molecular weight. Aminopeptidases from either origin have the same mol.wt. (280000) and the same sugar composition (23% by wt.) (Ugolev, 1972; Maroux et al., 1973; Malathi et al., 1973; Louvard et al., 1973, 1975a; Maroux & Louvard, 1976; Vannier et al., 1976).

The hydrophobic parts have a low mol.wt. (8000–10000) and, like lipids, are extractible by the chloroform/methanol mixture of Folch. Their high content of hydrophobic residues, in particular isoleucine and leucine, can explain this hydrophobic character. On the other hand, renal and intestinal aminopeptidase hydrophobic domains have the same composition in apolar residues, except that two valine and two tyrosine residues are missing from the renal peptide, which contains additional polar residues (Maroux & Louvard, 1976; Vannier et al., 1976). By contrast, the amino acid composition of the hydrophobic part of the maltases is completely different (Maroux & Louvard, 1976). Amino acid composition is thus characteristic of the type of hydrolase and not of the type of membrane.

These observations are important because if, as we think, aminopeptidase and maltases participate in transport, we can postulate that the hydrophobic part of the molecule could be involved in this process. If so, the structure and consequently the amino acid composition of this part of the molecule will have to be characteristic of the enzyme, as is indeed the case. Now we must ask if the position of the two parts of the hydrolases with respect to the lipid matrix of the membrane is compatible with this hypothesis of their direct involvement in the transport process.

Topological disposition of the aminopeptidases in the membrane

(1) In what proportions does the hydrophilic part emerge from the membrane? To answer this question, we have developed an immunological technique (Louvard et al., 1975b, 1976a). It is based on the fact that a protein antigen that is used as immunogen stimulates the production of antibodies directed specifically against exposed antigenic
determinants of its surface, and the maximum number of antibody molecules that can simultaneously bind this antigen corresponds to the total covering of the antigen surface. Thus antibodies are very good specific surface markers very suitable for the evaluation of the extent of integration of membrane proteins. By using $^{125}$I-labelled antibodies (Louvard et al., 1976a) against the trypsin form of aminopeptidase, it is easy to titrate the available determinants comparatively in the free (papain or trypsin form) and the membrane-bound (vesicles) enzymes. A constant amount of labelled antibody is incubated with various amounts of aminopeptidase, which is completely precipitated when the antibody/antigen molar ratio is greater than 2. After centrifugation, the radioactivity measured in the supernatant corresponds to the antibody excess. This value is plotted against the antibody/antigen molar ratio. As long as this ratio is lower than the ratio corresponding to the saturation of the antigen surface by its antibodies, no antibody is found in the supernatant. Above this ratio, detectable amounts of antibody are found in the supernatant and then increase linearly. Extrapolation of the straight line thus obtained gives the exact value of the ratio corresponding to the saturation. Steric hindrance due to the proximity of two molecules of aminopeptidase or to the proximity of the membrane could prevent the total occupancy of every accessible determinant on the bound enzyme. To account for this possibility, we used the depletion method instead: in a first step, the antibodies are incubated with a large excess of membrane-bound enzyme (1 mol of enzyme/mol of antibody). In these conditions there is a statistical occupancy of only one determinant per antigen molecule, and that avoids the problem of steric hindrance. After centrifugation the supernatant contains unbound antibodies specific for the determinants that are really accessible. These depleted antibodies are then used for direct titration, on the trypsin form of aminopeptidase, of the number of truly inaccessible determinants in the integrated enzyme. We found that only two determinants among the 12 and 10 antigenic determinants of the trypsin form of intestinal and renal aminopeptidases respectively are unable to bind antibodies in the membrane-bound enzyme. So at least 80% of the hydrophilic part of the renal and intestinal aminopeptidases emerges from the lipid matrix.

Using the same quantitative immunological method, we can show that the renal and intestinal aminopeptidases have six common determinants: the two masked in the membrane-bound enzymes and the four located in the active-site region (Vannier et al., 1976). Indeed, the intestinal enzyme can bind six molecules of anti-(renal aminopeptidase) and the renal enzyme can bind six molecules of anti-(intestinal aminopeptidase). Each enzyme binds the heterologous depleted antibodies specific for the two masked determinants. Fixation of antibody on the four common determinants accessible in the bound enzyme induced inactivation of the enzymes. These findings show the high degree of structural homology in the two most important regions of the hydrophilic part of the two enzymes.

(2) Does the hydrophobic part span the membrane? By using a preparation of intact brush border as starting material (Hopfer et al., 1973) closed right-side-out membrane vesicles can be obtained. It is easy to trap into these vesicles a high-molecular-weight reagent added to the medium while the vesiculation proceeds. Thus by using a photo-generated reagent, it is possible to specifically label the cytoplasmic space of the membrane.

Fig. 1 shows the experimental procedure for specifically labelling the hydrophobic part of the aminopeptidase, supposing that it spans the membrane (Louvard et al., 1976b). (i) The labelling reagent, the 4-fluoro-3-nitrophenyl azide, which can generate a nitrene by photolysis, is covalently attached to a macromolecule (human fragment Fab) before being trapped into the closed sealed vesicles from which it could not depart afterwards. (ii) After extensive washing, photolysis induces the cross-linking of the trapped reagent to accessible proteins. (iii) The membrane proteins are solubilized by detergent or papain. (iv) The human fragment Fab bearing the photogenerated reagent is labelled by its monospecific antibody coupled with peroxidase. (v) Aminopeptidase is then specifically precipitated by its antibody. The extent of the aminopeptidase photolabelling is
Fig. 1. Experimental procedure to label the part of aminopeptidase spanning the membrane

AP, aminopeptidase; Fab\textsubscript{H}, Fab fragment from human IgG (immunoglobulin G), myeloma protein Zeg; NAP-, 2-nitro-4-azidophenyl group; NAP-FAB\textsubscript{H}, 2-nitro-4-azidophenyl group covalently attached to Fab\textsubscript{H}; anti-AP, IgG anti-('papain form' of aminopeptidase); anti-Fab-PO, sheep Fab anti-(human IgG) labelled with peroxidase.


Table 1. Photolabelling of aminopeptidase from the inside of vesicles with fluoronitrophenyl azide and human fragment Fab

Control labelling from the outside was measuring by putting sealed vesicles which had not trapped reagent in vesiculization medium containing reagent, and photolysing them after extensive washing. A value for the hydrophobic part was obtained by subtracting fraction I or III from fraction II. NAP–Fab\textsubscript{H}, 2-nitro-4-azidophenyl group covalently attached to human Fab fragment.

<table>
<thead>
<tr>
<th>NAP–Fab\textsubscript{H} in the immunoprecipitate (ng/μg of aminopeptidase)</th>
<th>Labelling from the inside</th>
<th>Control labelling from the outside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain-treated form (I)</td>
<td>0.675 ± 0.03</td>
<td>—</td>
</tr>
<tr>
<td>Detergent-solubilized form (II)</td>
<td>1.95 ± 0.09</td>
<td>0.880 ± 0.02</td>
</tr>
<tr>
<td>Detergent-solubilized form, papain treated (III)</td>
<td>0.695 ± 0.03</td>
<td>0.800 ± 0.02</td>
</tr>
<tr>
<td>Hydrophobic part (IV)</td>
<td>1.27 ± 0.1</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

1977
determined by peroxidase activity in the precipitate (level of detection 1 fmol). Specific labelling of the hydrophobic part corresponds to the difference between the labelling of aminopeptidase after detergent and papain solubilization.

Table 1 gives the results obtained. The extent of labelling of the papain-treated form, in other words of the hydrophilic part, is very low and corresponds to the unspecific labelling due to the small amount of reagent that cannot be displaced from the outside membrane by extensive washing (control labelling from the outside). The hydrophobic part is significantly more labelled. Thus we must conclude that intestinal aminopeptidase is a transmembrane protein.

This may have several important biological implications, for instance, participation in the transport of amino acids; but also an eventual interaction with other cytoplasmic constituents of the cell could play a role of primary importance in the biogenesis and differentiation of the brush-border membrane.


The Tricarboxylate Carrier of the Mitochondrial Membrane: Solubilization and Partial Purification of Citrate-Binding Protein from Submitochondrial Particles

F. PALMIERI, G. GENCHI, I. STIPANI, P. RICCIO and E. QUAGLIARIELLO

Department of Biochemistry, University of Bari, 70126 Bari, Italy

The inner membrane of mitochondria contains specific carrier systems which may play a role in the regulation of metabolic pathways requiring the participation of both mitochondrial and cytoplasmic enzymes. Several pieces of evidence indicate that the transport of citrate through the mitochondrial membrane is a carrier-mediated process (Robinson, 1973; Fonyo et al., 1976). The citrate carrier catalyses an exchange between citrate and malate. The stoicheiometry of the reaction is 1:1 (Palmieri & Quagliaariello, 1968). Other substrates for this carrier are cis-aconitate, isocitrate and phosphoenolpyruvate. Under physiological conditions it is likely that cytosolic malate exchanges for intramitochondrial tricarboxylate or phosphoenolpyruvate. The carrier is virtually absent from mitochondria of specialized tissues where cytosolic fatty acid synthesis does not take place (Chappell, 1968). The transport of citrate has a very specific inhibitor, 1,2,3-benzenetricarboxylate, which has no inhibitory action on other transport systems (Quagliaariello & Palmieri, 1972). It is remarkable that the carrier is not inhibited by 1,2,4-benzenetricarboxylate and 1,3,5-benzenetricarboxylate (Kleineke et al., 1973), indicating that the carboxylate groups have to be the correct distance apart.

Vol. 5