BIOSYNTHESIS OF INTESTINAL MICROVILLAR PROTEINS

Putative precursor forms of microvillus aminopeptidase and sucrase–isomaltase isolated from Ca^{2+}-precipitated enterocyte membranes

E. Michael DANIELSEN, Hans SJÖSTRÖM and Ove NORÉN
Dept. of Biochemistry C, The Panum Institute, University of Copenhagen, 3 Blegdamsvej, 2200 Copenhagen N, Denmark

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1. Introduction

Pig small intestinal microvillus aminopeptidase (EC 3.4.11.2) and sucrase–isomaltase (EC 3.2.1.48, EC 3.2.1.10) are comprised of heterologous subunits [1–3]. However, when the source of these enzymes is the small intestine of an animal which 3 days prior to sacrifice has had its pancreatic duct disconnected, they each only consist of one type of polypeptide [2,3]. It has therefore been suggested that pancreatic proteases under normal in vivo conditions play a role as modifiers of the intestinal brush border enzymes [2,3].

Interest has focused on the existence of intracellular precursor forms of the brush border enzymes; thus, by using pulse labelling techniques under in vivo conditions, a high M_r single chain putative precursor of sucrase–isomaltase associated with the Golgi and basolateral membrane fractions has been shown in the rat [4]. In contrast, using the same animal, a rapid labelling of a soluble cytosol sucrase observed was suggested not to be associated with isomaltase [5]. Similarly for microvillus aminopeptidase, a soluble, cytosol form of the enzyme was found most rapidly labelled during in vivo pulse labelling experiments [6].

This paper reports the finding of new molecular forms of pig microvillus aminopeptidase and sucrase–isomaltase associated with intracellular and/or basolateral membranes of the enterocyte.

2. Experimental

The sources of all chemicals were those in [2,3]. Immunoadsorbents were prepared as in [2,3]. Preparation of rabbit antibodies against denatured pig microvillus aminopeptidase and isomaltase will be described elsewhere.

Pig small intestines were kindly delivered by the Dept. of Experimental Pathology, Rigshospitalet, Copenhagen. (If not otherwise stated, all procedures were done at 4°C.)

2.1. *Preparation of Ca^{2+} precipitable membranes*

Portions of frozen, everted and washed small intestinal pieces (150 g) were thawed in 500 ml 12 mM Tris–HCl (pH 7.1) containing 300 mM mannitol. Epithelial cells were separated from the intestinal pieces by vigorous vibration for 90 s followed by filtration through a Büchner funnel. The filtrate was diluted with 2.5 vol. deionized water and homogenized in a Kenwood blender for 30 s. The homogenate was centrifuged at 2500 × *g*, 15 min, and the supernatant was collected and made 10 mM in $CaCl_2$. After 20 min with frequent stirring, the preparation was centrifuged at 1500 × *g*, 15 min. The pellet was resuspended in ~200 ml ice-cold 50 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ by using a Potter-Elvehjelm homogenizer and centrifuged again as above. The washing was repeated once more with the $CaCl_2$-containing buffer followed by a washing in 50 mM Tris–HCl (pH 8.0). After the final washing, the preparation was pelleted by centrifugation at 4000 × *g*, 15 min. The pellet (Ca^{2+}-precipitated membrane fraction) was either stored at −20°C or immediately resuspended in 200 ml 50 mM Tris–HCl (pH 8.0) and solubilized by the addition of Triton X-100 up to 1%. After ≥1 h at 4°C with magnetic stirring, the suspension was centrifuged at 48 000 × *g*, 1 h and the supernatant was either

stored at −20°C or directly used in immunoadsorbent chromatography.

In one experiment, aprotinin (2.8. mg/l) was added to all buffers used in the preparation. In another experiment, the final pellet of Ca^{2+}-precipitated membranes was incubated at 37°C, 1 h before solubilisation with Triton X-100.

2.2. *Immunoadsorbent chromatography*

Anti-microvillus aminopeptidase–Sepharose 4B (2–3 ml) and anti-sucrase–isomaltase–Sepharose 4B were packed in 5 ml syringes and washed extensively with 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl and 0.1% Triton X-100. The solubilised Ca^{2+}-precipitated membrane fraction was passed through either of the immunoadsorbent columns at 4°C at a maximum flow-rate of 5 ml/h. The columns were then washed with ⩾10 vol. starting buffer before hypotonic elution at room temperature with 2 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100 was started. Enzymically active eluate fractions were pooled, concentrated by ultrafiltration and stored at −20°C until use.

2.3. *Polyacrylamide gel electrophoresis*

Gel electrophoresis in SDS–10% polyacrylamide gels was done as in [7]. Prior to electrophoresis, samples were denatured by boiling for 5 min in the presence of 1% SDS and 2.5% 2-mercaptoethanol. The following M_r indicators were used: pro-sucrase–isomaltase (260 000); subunits A (162 000) and B (123 000) of microvillus aminopeptidase; albumin (67 000); and ovalbumin (45 000). After electrophoresis protein bands were stained for protein with Coomassie brilliant blue or for carbohydrate by the periodic acid/Schiff reagent [8].

2.4. *Immunoelectrophoresis*

Charge-shift crossed immunoelectrophoresis was done as in [9]. First-dimensional polyacrylamide gel electrophoresis [7] followed by 2nd-dimensional immunoelectrophoresis against anti-denatured microvillus aminopeptidase or anti-denatured isomaltase was done as follows: After electrophoresis in the first dimension, an ~1 mm wide lane of polyacrylamide gel was longitudinally excised and placed in a 3–5 mm wide ditch on the immunoplate, below the antibody containing gel. Contact with the surrounding agarose gel was achieved by filling up the ditch with agarose. Second-dimension electrophoresis was run at 2–3 V/cm for 20 h. Precipitates were visualized by staining with Coomassie brilliant blue.

2.5. *Other methods*

Protein was determined by using the Bio-Rad protein assay (microassay procedure) (Bio-Rad Lab., Munich) using bovine serum albumin as standard. Enzyme activities were determined as in [2].

3. Results and discussion

Triton X-100 quantitatively (80–95%) solubilized aminopeptidase and sucrase–isomaltase from the Ca^{2+}-precipitated membrane preparation. In the subsequent purification, both enzymes were totally bound to the respective immunoadsorbents. Like the corresponding microvillar enzymes, the Ca^{2+} pellet aminopeptidase and sucrase–isomaltase were eluted with good yield by a hypotonic buffer [2,3]. Fig.1A shows the polypeptide composition of aminopeptidase and sucrase–isomaltase purified from the Ca^{2+}-precipitated membrane fraction compared to those obtained from the microvillus membrane of normal animals and animals having had their pancreatic duct disconnected 3 days prior to the sacrifice. The Ca^{2+} pellet aminopeptidase consist of polypeptides of app. M_r 166 000, 142 000

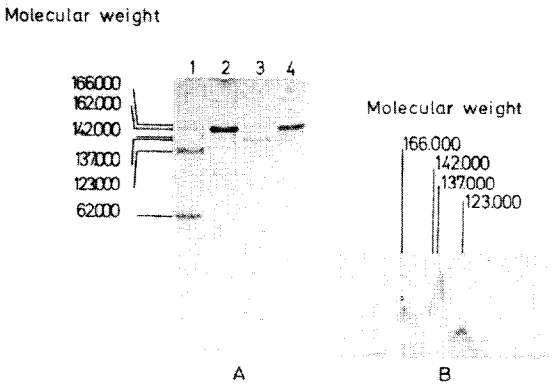

Fig.1. (A) SDS–Polyacrylamide gel electrophoresis of Ca^{2+} pellet aminopeptidase (3) and microvillus aminopeptidase from a normal animal (1) and from an animal with disconnected pancreatic duct (2,4). Protein (10–25 μg) was applied to each lane and after electrophoresis, the gel was stained for protein with Coomassie brilliant blue. (B) First-dimensional polyacrylamide gel electrophoresis followed by 2nd-dimensional immunoelectrophoresis of Ca^{2+} pellet aminopeptidase against anti-denatured microvillus aminopeptidase (B subunit). The IgG content of the gel was 0.25 mg/ml and ~1 μg aminopeptidase was applied.

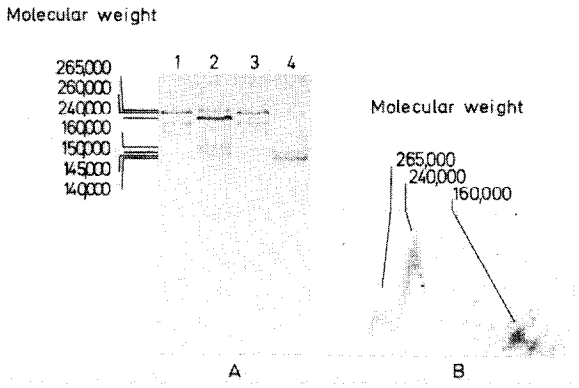

Fig.2. (A) SDS–Polyacrylamide gel electrophoresis of Ca^{2+} pellet sucrase–isomaltase (2) and microvillus sucrase–isomaltase from a normal animal (4) and from an animal with disconnected pancreatic duct (1,3). Protein (10–25 μg) was applied to each lane and after electrophoresis, the gel was stained for protein with Coomassie brilliant blue. (B) First-dimensional polyacrylamide gel electrophoresis followed by 2nd-dimensional immunoelectrophoresis of Ca^{2+} pellet sucrase–isomaltase against anti-denatured microvillus isomaltase. The IgG content of the gel was 0.25 mg/ml and ~1 μg sucrase–isomaltase was applied.

and 137 000, all of which are glycosylated as judged by positive reaction with the periodic acid/Schiff reagent. The upper band migrates somewhat slower than the single polypeptide observed in aminopeptidase prepared from the microvillus membrane of animals with disconnected pancreatic duct (also when the two enzymes are electrophoresed in the same lane). In addition, components corresponding to the 2 lower subunits of the microvillus aminopeptidase were detectable. All 4 upper bands of the Ca^{2+} pellet aminopeptidase gave rise to immunoprecipitates in the 2nd-dimensional immunoelectrophoresis against antibodies to denatured microvillus aminopeptidase (fig.2B). The fusion of the precipitates indicates immunochemical cross-reactivity between the respective polypeptide components. (The antibodies used were directed against the 123 000 M_r polypeptide of the microvillar enzyme which cross-reacts with the 162 000 but not with the 62 000 M_r polypeptide).

The principal band of the Ca^{2+} pellet sucrase–isomaltase has app. M_r 240 000, but a band moving slightly slower (M_r 265 000) than the single-chain microvillar pro-sucrase–isomaltase (also when electrophoresed in the same lane) is also clearly visible (fig.2A). Both bands gave a positive reaction with the periodic acid/Schiff reagent. In addition, two blurred bands of M_r 160 000 and 145 000 can be detected. The two upper bands produced fusing immunoprecipitates when run against antibodies to denatured isomaltase (fig.2B). Also the polypeptide of M_r 160 000 reacted with this antibody preparation.

The polypeptide composition of both enzymes studied appears strikingly different compared to that normally obtained from the microvillus membrane. The immunoelectrophoretic experiments show that even the faint bands of Ca^{2+} pellet aminopeptidase and sucrase–isomaltase are structurally related to the corresponding microvillar enzymes. For aminopeptidase, the usually most abundant polypeptides of M_r 123 000 and 62 000 are barely visible and the 2 lower bands of sucrase–isomaltase, supposedly representing separated sucrase and isomaltase subunits, but with mobilities different from those of the microvillus membrane, are also of relative minor intensity. For both enzymes, the most slowly migrating polypeptide is almost in position with that from the microvillus membrane of animals with disconnected pancreatic duct, indicating that only minor modifications will produce the final enzyme.

The 142 000 and 137 000 M_r bands of aminopeptidase and the 240 000 M_r band of sucrase–isomaltase cannot be easily related to any of the polypeptides of the final microvillar enzymes. The possibility that they are preparative artefacts cannot a priori be excluded but must be considered highly unlikely on several grounds. Pancreatic proteolysis during preparation would as shown [2,3] produce the 2 lower bands of aminopeptidase and split pro-sucrase–isomaltase into the 2 subunits of 'final' sucrase–isomaltase. Neither the presence of aprotinin during the entire preparation nor incubation of the Ca^{2+} pellet at 37°C prior to solubilisation changed the polypeptide composition of any of the enzyme preparations. Finally, in charge-shift crossed immunoelectrophoresis, both Ca^{2+} pellet aminopeptidase and sucrase–isomaltase appeared electrophoretically heterogeneous but significantly exhibited both cationic and anionic shifts, indicating that the site usually most susceptible to proteolytic cleavage on integral stalked proteins, the region between the hydrophilic head group and the anchor, was intact.

These data do not allow an exact cellular localization of the various forms of aminopeptidase and sucrase–isomaltase found in the Ca^{2+} pellet. The Ca^{2+} (or Mg^{2+}) precipitation of microsomal fractions is widely used as the key step in the preparation of

microvilli from intestine [10] and kidney [11]. However, a considerable amount of microvillar vesicles in the Mg^{2+}-precipitated fraction was observed when using neutral endopeptidase as a marker enzyme [11]. We found ~6–9% of the total microvillus aminopeptidase activity in the first Ca^{2+}-precipitate, but the repeated washings reduced this to 3–4% without significantly decreasing the size of the pellet. We therefore believe that the washings remove small amounts of microvillar vesicles initially entrapped in the bulky Ca^{2+} pellet, which contains as much as 12% of the total cellular protein.

The Ca^{2+}-precipitated fraction, as expected, contains membrane vesicles of mitochondrial, endoplasmic and basolateral origin. Mitochondria are not known to contain either of the enzymes studied, but endoplasmic reticulum as well as the basolateral membrane could be the cellular source of the Ca^{2+} pellet forms of aminopeptidase and sucrase–isomaltase. According to the membrane flow hypothesis of membrane biogenesis [12] (not yet demonstrated to be applicable to the microvillus membrane) newly synthesized forms of the microvillar enzymes should be associated with the endoplasmic reticulum. In the special case of the enterocyte, it has been suggested that newly synthesized proteins, finally destined for the microvillus membrane, are initially transported to the basolateral portion of the plasma membrane [13,14]. The fact that both aminopeptidase and sucrase–isomaltase have been shown to be present in this membrane lends support to this view [4,15].

The various forms of aminopeptidase and sucrase–isomaltase found in the Ca^{2+}-precipitated membrane fraction may well represent different stages in the biogenesis of the corresponding, mature microvillar enzymes. Thus, in [16] two intracellular forms of the G protein of vesicular stomatitis virus were distinguished, differing only in their carbohydrate content; one 'high-mannose' form, representing the G protein in its state of transport in coated vesicles from the endoplasmic reticulum to the Golgi apparatus and a second 'complex' form, representing the mature form of the protein transported from the Golgi apparatus to the plasma membrane. The relative small differences in M_r between the various polypeptide components of the Ca^{2+} pellet aminopeptidase and sucrase–isomaltase could hypothetically be accounted for by differences in glycosylation. To examine this further, more information about the cellular origin of the individual polypeptide components and the nature of differences in their M_r is needed. Such studies are in progress in our laboratory.

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