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Biosynthesis of intestinal microvillar proteins

Pulse–chase labelling studies on maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV

E. Michael DANIELSEN, Hans SJÖSTRÖM and Ove NORÉN
Department of Biochemistry C, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

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The biogenesis of three intestinal microvillar enzymes, maltase–glucoamylase (EC 3.2.1.20), aminopeptidase A (aspartate aminopeptidase, EC 3.4.11.7) and dipeptidyl peptidase IV (EC 3.4.14.5), was studied by pulse–chase labelling of pig small-intestinal explants kept in organ culture. The earliest detectable forms of the enzymes were polypeptides of M_r 225000, 140000 and 115000 respectively. These were found to represent the enzymes in a 'high-mannose' state of glycosylation, as judged by their susceptibility to treatment with endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96). After about 40–60min of chase, maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV were further modified to yield the mature polypeptides of M_r 245000, 170000 and 137000 respectively, which were expressed at the microvillar membrane after 60–90min of chase. The fact that the enzymes before reaching the microvillar membrane were found in a Ca^{2+}-precipitated membrane fraction (intracellular and basolateral membranes), but not in soluble form, indicates that during biogenesis maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV are transported and assembled in a membrane-bound state.

The intestinal microvillar enzymes maltase–glucoamylase (EC 3.2.1.20), aminopeptidase A (aspartate aminopeptidase, EC 3.4.11.7) and dipeptidyl peptidase IV (EC 3.4.14.5) have all been purified and characterized in their amphiphilic form (Sørensen *et al.*, 1982; Colas & Maroux, 1980; Svensson *et al.*, 1978). However, little is so far known about the biogenesis of these enzymes. For maltase–glucoamylase, it has been shown that it exists as a large single-chain polypeptide in animals with surgically ligated pancreatic ducts (Sørensen *et al.*, 1982), and a 'high-mannose' glycosylated polypeptide of the enzyme has been shown to be present in a Ca^{2+}-precipitated membrane fraction of enterocytes (Danielsen *et al.*, 1981). In organ-cultured explants of pig small intestine, maltase–glucoamylase and aminopeptidase A were both radioactively labelled during continuous incubation with [^{35}S]methionine for 24h, and the labelled enzymes had M_r values comparable with previously published values (Danielsen *et al.*, 1982*a*). The organ-culture system was found to be suitable for performing pulse–chase labelling studies on two other microvillar enzymes, aminopeptidase N (EC 3.4.11.2) and sucrase (EC 3.2.1.48)–isomaltase (EC 3.2.1.10) (Danielsen, 1982).

In the present work, pulse–chase labelling studies were performed with organ-cultured intestinal explants to follow kinetically the biogenesis of maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV and to examine the post-translational modifications occurring during this process.

Materials and methods

Materials

Pig small intestines were kindly given by the Department of Experimental Pathology, Rigshospitalet, Copenhagen, Denmark.

Materials and equipment for performing organ culture, including Trowell's T-8 medium, foetal-calf serum, sterile plastic dishes with grids and [^{35}S]-methionine (sp. radioactivity > 1000Ci/mmol) were obtained as previously described (Danielsen *et al.*, 1982*a*).

The sources of specific rabbit antibodies to maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV were as described previously (Sørensen *et al.*, 1982; Danielsen *et al.*, 1980; Svensson *et al.*, 1978).

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Organ culture

Small-intestinal explants were excised and placed in Falcon dishes for organ culture as previously described (Danielsen *et al.*, 1982*a*). Before starting the pulse–chase experiments, explants were cultured in the non-radioactive medium for 1–2h. They were then pulse-labelled for 10min with [^{35}S]methionine (200–500μCi/ml) and chased with non-radioactive medium for various periods of time as previously described (Danielsen, 1982). In some experiments, the chase medium contained an increased concentration of methionine (2.5mM). Explants used for endo-β-*N*-acetylglucosaminidase H experiments were labelled continuously for 90min with 100μCi of [^{35}S]methionine/ml. The culture was stopped by washing the explants with 3 x 1ml of ice-cold 0.9% NaCl. Immediately after, the explants were frozen at −80°C, at which temperature they were kept until further processing.

Fractionation of labelled explants

After culture, pulse-labelled explants were fractionated into a Ca^{2+}-precipitated membrane fraction, a microvillar fraction and a soluble fraction as described by Danielsen (1982). Before the purification of the microvillar enzymes by immunoelectrophoresis, membrane-bound protein was solubilized by Triton X-100 as previously described (Danielsen, 1982).

Immunopurification of the enzymes

The microvillar enzymes were purified from labelled explants by line immunoelectrophoresis (Axelsen *et al.*, 1973) against specific antibodies as previously described (Danielsen *et al.*, 1982*a*; Danielsen, 1982).

Treatment with endo-β-N-acetylglucosaminidase H

Samples (100μl) of immunopurified aminopeptidase A and dipeptidyl peptidase IV in 0.1M-sodium citrate, pH6.5, containing 0.1% (w/v) sodium dodecyl sulphate, were boiled for 3min and incubated with 7munits of endo-β-*N*-acetylglucosaminidase H as previously described (Danielsen, 1982). Control samples without addition of glycosidase were incubated in parallel.

Polyacrylamide-gel electrophoresis

Gel electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulphate was performed as described by Laemmli (1970). Before electrophoresis, samples were denatured by boiling for 5min in the presence of 1% sodium dodecyl sulphate and 2.5% 2-mercaptoethanol. The following M_r indicators were used: polypeptides (M_r 140000 and 166000) of aminopeptidase N (Danielsen, 1982), polypeptides (M_r 225000 and 245000) of maltase–glucoamylase (Danielsen *et al.*, 1981) and microvillar dipeptidyl peptidase IV (M_r 137000) (Svensson *et al.*, 1978). After electrophoresis, the gels were prepared for fluorography as described by Bonner & Laskey (1974).

Kodak X-Omat films were exposed at −80°C.

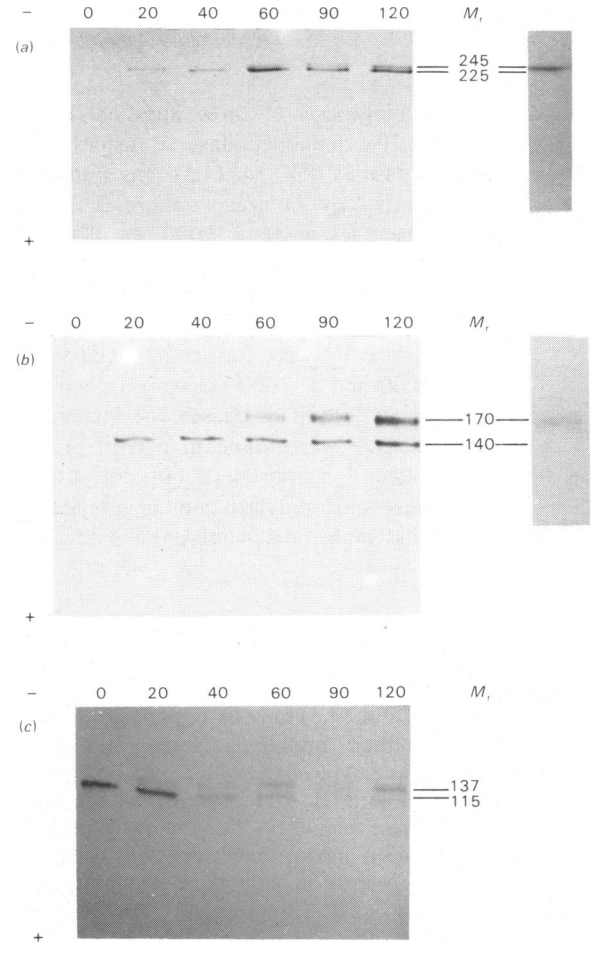

Fig. 1. *Pulse–chase labelling of maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV from the solubilized Ca^{2+}-precipitated membrane fraction*

After 10min of labelling, explants were chased for the indicated periods of time (min): (*a*), maltase–glucoamylase; (*b*), aminopeptidase A; (*c*), dipeptidyl peptidase IV. For maltase–glucoamylase and aminopeptidase A, the lane furthest to the right shows the labelling of the enzymes, isolated from a Triton X-100 extract of total explant protein (Danielsen, 1982), when the chase medium contained 2.5mM-methionine. In the experiment with dipeptidyl peptidase IV, the chase medium also contained 2.5mM-methionine. Apparent M_r values ($\times 10^{-3}$) are shown. Exposure times: 20–30 days.

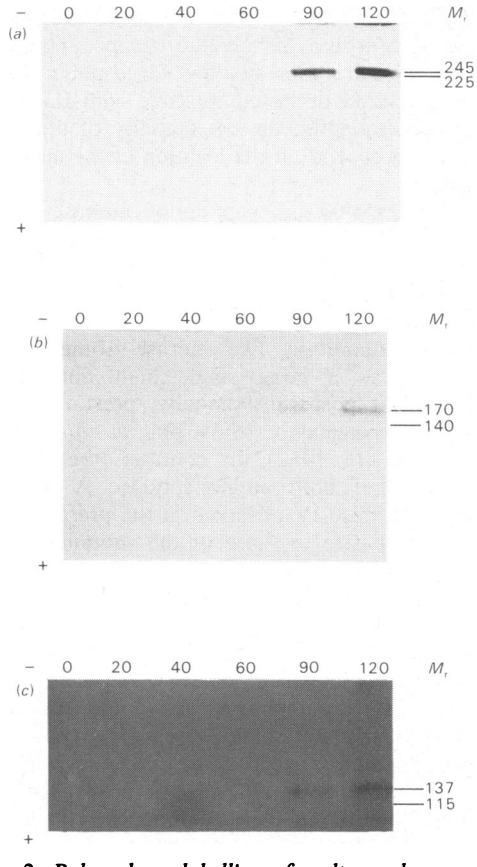

Fig. 2. *Pulse–chase labelling of maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV from the solubilized microvillar membrane fraction*

After 10 min of labelling, explants were chased for the indicated periods of time (min): (*a*), maltase–glucoamylase; (*b*), aminopeptidase A; (*c*), dipeptidyl peptidase IV. Apparent M_r values ($\times 10^{-3}$) are shown. Exposure times: 20–30 days.

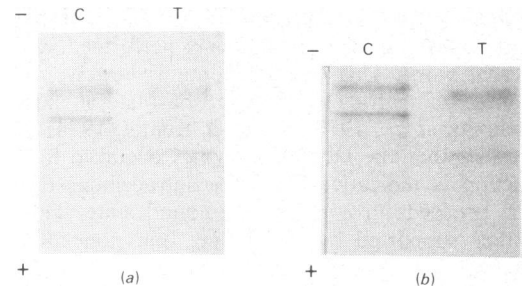

Fig. 3. *Susceptibility of labelled aminopeptidase A and dipeptidyl peptidase IV to treatment with endo-β-N-acetylglucosaminidase H*

After 90 min of labelling, organ culture was stopped, and aminopeptidase A (*a*) and dipeptidyl peptidase IV (*b*) were immunopurified from a Triton X-100 extract of total explant protein (Danielsen, 1982). T and C indicate endo-β-*N*-acetylglucosaminidase H-treated and control samples, respectively. Exposure time: 5 days.

Results

Fig. 1 shows the appearance in the Ca^{2+}-precipitated membrane fraction of [^{35}S]methionine-labelled maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV, after a 10 min pulse period. For maltase–glucoamylase, a polypeptide of M_r 225000 was clearly visible after 20 min of chase, but it could only be detected immediately after the pulse after prolonged exposure of the fluorograph. A second polypeptide, of M_r 245000, appeared after 60 min of chase. For aminopeptidase A, the first polypeptide, visible after 20 min of chase, was of M_r 140000 (again, this polypeptide was also detectable immediately after the pulse after prolonged exposure of the fluorograph), followed by a polypeptide of M_r 170000 after 40 min of chase. Finally, for dipeptidyl peptidase IV, the first polypeptide appearing immediately after the pulse was of M_r 115000. A second polypeptide, of M_r 137000, was visible after 40 min of chase. For all three enzymes, only the polypeptide of higher M_r significantly persisted after 90–120 min of chase when the chase medium contained an increased concentration of methionine.

The appearance in the microvillar membrane of the labelled enzymes is shown in Fig. 2. For all three enzymes, only one radioactive polypeptide was observed in this membrane fraction, and these only after 60–90 min of chase. In each case the labelled form corresponded to the higher-M_r form observed in Fig. 1. Neither maltase–glucoamylase, aminopeptidase A nor dipeptidyl peptidase IV could be detected in the soluble fraction within 0–120 min of chase (results not shown).

Fig. 3 shows the effect of endo-β-*N*-acetylglucosaminidase H on labelled explant aminopeptidase A and dipeptidyl peptidase IV. For aminopeptidase A, the polypeptide of M_r 170000 was only slightly affected by the treatment with the glycosidase, whereas the polypeptide of M_r 140000 exhibited a significant shift in electrophoretic mobility. For dipeptidyl peptidase IV, only the polypeptide of M_r 115000 was susceptible to treatment with endo-β-*N*-acetylglucosaminidase H.

Discussion

Maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV first appeared radioactively

labelled in the Ca^{2+}-precipitated membrane fraction in the pulse-labelling experiments performed in the present work. This fraction contains largely membranes of intracellular and basolateral origin (Schmitz *et al.*, 1973; Booth & Kenny, 1974), and the fact that the labelled enzymes occurred in this fraction is indicative that their intracellular transport proceeds in a membrane-bound state. This is further supported by the finding that none of the labelled enzymes was detectable in soluble form during the entire chase period of 120 min. For maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV, the early-labelled polypeptides of lower M_r (225000, 140000 and 115000 respectively) were not expressed in the microvillar membrane. There are good reasons to assume that these forms of the enzymes represent transient, exclusively intracellular, precursor forms of the final enzymes, which are post-translationally converted into the polypeptides of higher M_r (245000, 170000 and 137000 for maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV respectively) that occur in the Ca^{2+}-precipitated membrane fraction after 40–60 min of chase and in the microvillar membrane fraction after 60–90 min of chase. Firstly, the polypeptides of lower M_r did not significantly persist in the Ca^{2+}-precipitated membrane fraction when the chase medium contained increased concentrations of methionine. Secondly, for aminopeptidase A and dipeptidyl peptidase IV, the polypeptides of M_r 140000 and 115000, respectively, were found to be greatly susceptible to the action of endo-β-*N*-acetylglucosaminidase H, suggesting that they contain the 'high-mannose' glycosylation which is characteristic of intermediate glycosylation for proteins destined for cellular externalization (Robbins *et al.*, 1977). In contrast, the higher-M_r polypeptides of aminopeptidase A and dipeptidyl peptidase IV were only slightly affected or were insensitive to treatment with the glycosidase. For maltase–glucoamylase, a polypeptide of M_r 225000, prepared on preparative scale from a Ca^{2+}-precipitated membrane fraction of enterocytes, was previously found to be susceptible to treatment with endo-β-*N*-acetylglucosaminidase H (Danielsen *et al.*, 1981). Finally, it has been shown that, for maltase–glucoamylase and aminopeptidase A, only the polypeptides of higher M_r accumulate when explants are cultured for 24 h (Danielsen *et al.*, 1982*a*).

The fact that for maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV, the earliest detectable form of the enzymes was 'high-mannose' glycosylated indicates that this glycosylation, for all three enzymes, occurs during or immediately after the translation process. The short (10 min) pulse period may explain why the polypeptides of maltase–glucoamylase and aminopeptidase A could be observed immediately after the pulse only after prolonged exposure of the fluorograph. The time required for translation of these large polypeptides may well exceed or be comparable with the pulse period; consequently, the detectability of the polypeptides will depend on the location of the methionine residues.

The M_r-245000 polypeptide of maltase–glucoamylase is equal in size to that of the enzyme, obtained from a microvillar fraction of pigs with ligated pancreatic ducts (Sørensen *et al.*, 1982). The present work thus confirms the earlier finding that maltase–glucoamylase, like sucrase–isomaltase, is synthesized as a large single-chain polypeptide which is split post-translationally, presumably by pancreatic proteinases (Sjöström *et al.*, 1980; Sørensen *et al.*, 1982). In contrast, the labelled polypeptides of both aminopeptidase A and dipeptidyl peptidase IV observed in the present work correspond in size to those of the subunits of the respective enzymes (Danielsen *et al.*, 1982*a*; Svensson *et al.*, 1978). (For aminopeptidase A, two faint bands in positions corresponding to twice the size of the M_r-170000 and -140000 polypeptides could be seen. However, since such bands were visible in the Ca^{2+}-precipitated membrane fraction and the microvillar fraction, they cannot be indicative of the existence of a large single-chain precursor, but are interpreted as dimers, not dissociated by the pretreatment before the electrophoresis.) The two peptidases studied in the present work therefore resemble aminopeptidase N, which in pulse-labelling studies was found initially to appear intracellularly as a M_r-140000 polypeptide (Danielsen, 1982) and in a cell-free translation system to be synthesized as a M_r-115000 polypeptide (Danielsen *et al.*, 1982*b*), indicating that the mRNA-translation product of this enzyme is of the subunit size of the microvillar molecule.

The results obtained in the present work on maltase–glucoamylase, aminopeptidase A and dipeptidyl peptidase IV are parallel to those described for aminopeptidase N and sucrase–isomaltase in a similar study (Danielsen, 1982). The similarities in the kinetics of the biogenesis of these five microvillar enzymes in the organ-culture system makes it tempting to assume the existence in the enterocyte of a common transport route for all proteins destined for insertion in the microvillar membrane. The fact that the enzymes undergo the same type of post-translational glycosylation supports this hypothesis; the early, membrane-bound, 'high-mannose' glycosylated form of the enzymes is probably indicative of the site of synthesis being the rough endoplasmic reticulum, and the intracellular re-glycosylation to yield the mature form of the enzymes may well take place in the Golgi complex, which is the site of fucosylation of membrane proteins in intestinal

epithelial cells (Quaroni *et al.*, 1979). Evidence so far therefore suggests that the biogenesis of intestinal microvillar enzymes occurs in accordance with the general 'membrane flow' hypothesis (Palade, 1975).

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