Immunochemical characterization and biosynthesis of pI-6.4 esterase, a carboxylesterase of rat liver microsomal extracts

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Rat liver pI-6.4 esterase was purified from microsomes (microsomal extracts) and used to generate antibodies in the rabbit. Two active enzyme forms, similarly sensitive to endo-H (endo- β -N-acetylglucosaminidase H (EC 3.2.1.96), but differing slightly in polypeptide chain length, were present in the preparation. In microsomes, immunoblots revealed a single form, with $M_r \simeq 62000$, identical with the large component of the purified enzyme, indicating that the second component is an artefact. Rabbit reticulocyte lysates and wheat-germ extracts programmed with RNA extracted from total or bound polysomes synthesized a single immunoreactive 61 kDa polypeptide, which was not formed with RNA extracted from free polysomes. The immunoreactive product synthesized in the presence of dog pancreas microsomes was slightly larger (62 kDa); like the authentic enzyme, it bound to concanavalin A and was decreased in molecular size to 60 kDa by the action of endo-H. Thus the enzyme is synthesized with a short cleavable sequence and bears at least one high-mannose oligosaccharide chain. Metabolic labelling in hepatocytes cultured with [35 S]methionine also generated a single immunoreactive polypeptide of 62 kDa, which was decreased to 60 kDa in size by treatment with endo-H or addition of tunicamycin to the culture medium. This confirms the molecular homogeneity and the glycosylation of the enzyme in the intact cell. Culture media contained no pI-6.4-esterase-related protein, whether tunicamycin was present or not. The processing steps in the synthesis of pI-6.4 esterase are thus, as for other esterases of the endoplasmic reticulum [Robbi & Beaufay (1986) *Eur. J. Biochem.* **158**, 187–194; (1987) *Biochem. J.* **248**, 545–550] indistinguishable from those occurring early in the synthesis of secretory proteins. Glycosylation is apparently not the sorting signal responsible for their retention in the endoplasmic reticulum.

INTRODUCTION

Proteins resident in the lumen of the endoplasmic reticulum (ER) are sorted out of the traffic of secretory proteins. How they acquire their stable localization in the ER is still far from being understood. Sorting mediated by a signal for retention in the ER seems now more likely than a purely passive process resulting from the lack of a signal for exit (discussed by Rothman, 1987). The microsomal β -glucuronidase is retained by egasyn, a protein of the ER with esterase activity (Medda *et al.*, 1987*a,b*). The C-terminal sequence: -Lys-Asp-Glu-Leu functions as a topogenic signal in three proteins of the ER lumen, but the subcellular component that recognizes the sequence is apparently not the ER membrane (Munro & Pelham, 1987). Another short C-terminal stretch of amino acid residues exposed on the cytoplasmic side of the ER membrane is responsible for the retention of the E19 transmembrane glycoprotein of adenoviruses in the ER (Pääbo *et al.*, 1987).

In rat liver, esterase activity (EC 3.1.1.1) is localized in the ER (Amar-Costesec *et al.*, 1974; Beaufay *et al.*, 1974*b*) and shared by several isoenzymes, which are either free in the lumen or loosely bound to the inner aspect of the membrane (Robbi & Beaufay, 1983). They are thus good model proteins to investigate the

mechanism of protein retention in the ER lumen. Their primary structure is encoded in four different genes (Simon *et al.*, 1985). They are glycosylated, some show structural microheterogeneity (Mentlein *et al.*, 1980; Robbi & Beaufay, 1983; Simon *et al.*, 1985), and all migrate in SDS/polyacrylamide gels as $\simeq 60$ kDa polypeptides. The native enzymes are monomers, except the pI-6.1 enzyme (ES-10A), which is a trimer (Mentlein *et al.*, 1980; Robbi & Beaufay, 1983). Among the attractive features for their study, these enzymes can be easily revealed in non-denaturing PAGE by histochemical staining (Ljungquist & Augustinsson, 1971) and specifically labelled by radioactive inhibitors. Their primary structure is still unknown, but the N- and C-terminal amino acids of several isoenzymes have been identified (Mentlein *et al.*, 1980, 1984*a*). The action of esterases on numerous foreign chemicals, including therapeutic drugs, and on natural lipids, has been extensively investigated by Heymann (1980) and Mentlein *et al.* (1984*b,c*).

The present paper is concerned with pI-6.4 esterase (genetic nomenclature ES-4B; Mentlein *et al.*, 1987). We show that the enzyme does not occur in different molecular forms, as was once believed (Mentlein *et al.*, 1980, 1984*a*; Robbi & Beaufay, 1983; Simon *et al.*, 1985), but that it is a well-defined 62 kDa N-glycosylated

Abbreviations used: endo-H, endo- β -N-acetylglucosaminidase H (EC 3.2.1.96); PAGE, polyacrylamide-gel electrophoresis; ER, endoplasmic reticulum.

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protein of the high-mannose type. We have investigated its biosynthesis in cell-free extracts and in primary cultures of hepatocytes. The results reveal many similarities with the biosynthesis of other esterases (Robbi & Beaufay, 1986, 1987).

EXPERIMENTAL

Materials

Adult Wistar rats, non-inbred strain Han:Wist (180–220 g) were purchased from Proefdiencentrum, Heverlee, Belgium; untoasted wheat germ was kindly provided by General Mills, Vallejo, PA, U.S.A.; endo-H and *Staphylococcus aureus* V8 proteinase were purchased from Miles, Naperville, IL, U.S.A., and *S. aureus* protein A was from Atlantic Antibodies, Scarborough, ME, U.S.A.

Methods

Purification of the pI-6.4 esterase. Esterases were isolated simultaneously from rat liver microsomes as described previously, up to the preparative-isoelectric-focusing step (Robbi & Beaufay, 1983). The fractions focusing around pH 6.4 were purified on Superose-12 and DEAE-Trisacryl, as described for the pI-6.1 enzyme (Robbi & Beaufay, 1987). Elution of esterase activity was monitored by using *o*-nitrophenyl acetate as substrate (Beaufay *et al.*, 1974a). The active fractions were subjected to PAGE, followed by histochemical staining with α -naphthyl acetate and Fast Blue RR to distinguish between the various esterase isoenzymes. At this stage the pI-6.4-esterase preparation was still appreciably contaminated with another protein. Final purification was achieved on a preparative 9%-(w/v)-polyacrylamide gel. After histochemical staining, the gel was excised to elute the enzyme with an Isco electrophoretic sample concentrator (Isco, Lincoln, NE 68505, U.S.A.). Electroelution was carried out in a cold-room in 12.5 mM-Tris/86 mM-glycine buffer, pH 8.3, for 15 h at 50 V. The current was reversed for 30 s before removal of the enzymes from the concentration wells.

Antibodies. The electrophoretically purified pI-6.4 esterase preparation was used to generate an antiserum in rabbit. The procedure was identical with the one used to produce the serum directed against pI-6.1 esterase (Robbi & Beaufay, 1987), except that an 8–10-fold lower amount of antigen was used for each injection.

Peptide maps. The procedure was adapted from that described by Cleveland *et al.* (1977). Immunoprecipitates of pI-6.4 esterase labelled *in vivo* and *in vitro* were analysed by SDS/PAGE and fluorography (Chamberlain, 1979). The bands localized on X-ray film were excised from the dried gel, soaked briefly in water to detach the filter paper, and transferred to the large well of the Isco electrophoretic concentrator. The proteins, eluted as described above, were recovered in the concentration well, extensively dialysed against water to remove residual sodium salicylate, and freeze-dried. The material was redissolved by boiling for 3 min in 30 μ l of 125 mM-Tris/HCl (pH 6.8)/0.1% SDS/20% (v/v) glycerol/1 mM-EDTA/0.3% 2-mercaptoethanol. Proteolysis was initiated by the addition of 2 μ l of *S. aureus* V8 proteinase (0.5 mg/ml in 125 mM-Tris/HCl, pH 6.8). After 30 min at room temperature, the enzyme was

Fig. 1. Analysis of the pI-6.4 esterase preparation by PAGE

(a) Is a zymogram: lane 1, proteins extracted from 200 μ g of microsomal protein with 0.2% Triton X-100; lane 2, purified preparation (≈ 0.75 μ g of protein). Isoelectric points (pI) of esterases given in the left margin were established by preparative isoelectric focusing (Robbi & Beaufay, 1983). (b) Is a silver-stained SDS/polyacrylamide gel loaded with the purified pI-6.4 esterase preparation (≈ 0.75 μ g of protein). The values on the left side indicate M_r values for the standards [serum albumin and ovalbumin (arrows)] and the purified pI-6.4 esterases (arrowheads).

inactivated by the addition of SDS, 2-mercaptoethanol and Bromophenol Blue up to 5, 2 and 0.01% respectively, and boiling for 2 min. The generated peptides were resolved on a 15%-(w/v)-polyacrylamide/SDS gel. After fixation in 15% (w/v) trichloroacetic acid, the 35 S-labelled peptides were revealed by fluorography in 1 M-sodium salicylate.

Other techniques. Immunotitration and immunoblotting, labelling with di-isopropyl fluorophosphate, preparation of cell-free systems for protein synthesis, preparation of polysomes and extraction of RNA, translation *in vitro*, cell culture and metabolic labelling, immunoprecipitation of newly synthesized polypeptides, adsorption on concanavalin A and treatment with endo-H, PAGE with or without SDS, and fluorography, were all performed as described previously (Robbi & Beaufay 1983, 1986, 1987). Silver staining was performed as described by Merril (1981) and protein determination was by the method of Bradford (1976), with bovine serum albumin as standard.

RESULTS

Analysis of the pI-6.4 esterase preparation

The electrophoretic behaviour of the pI-6.4 esterase preparation in non-denaturing polyacrylamide gels is depicted in Fig. 1(a) (lane 2). It shows two active enzyme forms, stained with α -naphthyl acetate and Fast Blue RR. Two protein bands were also seen by silver staining of a duplicate gel (results not shown). These two proteins co-migrate with two esterase-staining bands of the crude Triton X-100 extract, of which the slow one is prominent (lane 1).

Fig. 2. Immunotitration of the antiserum

Identical aliquots of a crude Triton X-100 extract from female-rat liver microsomes were incubated for 1 h at 26 °C in 100 µl of medium which contained 10 mM-phosphate buffer, pH 7.4, 0.15 M-NaCl, 1 % Triton X-100 and different amounts of antiserum directed against pI-6.4 esterases. The immune complexes were spun down (Eppendorf Microfuge, 5 min, 16000 g); the supernatants were analysed on a 9 % (w/v) acrylamide gel and stained for esterase activity. Isoelectric points (pI) of esterases given in the left margin were established by preparative isoelectric focusing.

Fig. 1(b) is a silver-stained SDS/polyacrylamide gel of the preparation. Again two polypeptides are seen, with apparent M_r values of 60000 and 62000. SDS/PAGE after incubation with [3 H]di-isopropyl fluorophosphate reveals that these two polypeptides react with the inhibitor (see Fig. 4 below, lane 2, and Fig. 5 below, lane 1).

Specificity of the antiserum

In consideration of previous observations indicating that the two components of the pI-6.4 esterase preparation are immunologically related, and because only a limited amount of electrophoretically purified protein was available, the mixture of both enzyme forms was used to generate antibodies in rabbit. Immunotitration of a crude Triton X-100 extract with this antiserum precipitates two closely migrating enzyme forms (Fig. 2), corresponding to the purified antigens Fig. 1(a).

When the specificity of this antiserum was further tested by an immunoblot of a crude Triton X-100 extract resolved by non-denaturing PAGE, antibodies directed against the pI-6.1 esterase could also be detected (results not shown). From protein-stained gels of partially purified preparations, we estimate that the pI-6.1 esterase is, in liver, more abundant by an order of magnitude than the pI-6.4 forms. Thus blots of crude extracts are more heavily loaded with pI-6.1 esterase and more

Fig. 3. Immunoblots of microsomal proteins allowed to react with the antiserum directed against pI-6.4 esterases

Proteins were resolved by PAGE in the absence (a) or in the presence (b) of SDS. They were then transferred to nitrocellulose, probed with a 2000-fold dilution of the antiserum preadsorbed on an affinity column of pI-6.1 esterase linked to Sepharose-4B, and developed with 125 I-protein A. Lanes 1, proteins extracted from microsomes with 0.2% Triton X-100 and partially purified by electrofocusing (pI 6.4); lanes 2, crude 0.2% Triton X-100 extracts (from 200 µg of microsomal protein); lane 3, microsomes (40 µg of protein).

Fig. 4. Endo-H treatment of purified pI-6.4 esterases

[3 H]Di-isopropyl fluorophosphate-labelled pI-6.4 esterases (0.75 µg of protein) were incubated for 18 h at 37 °C in the absence (lane 2) or presence (lane 3) of endo-H. [14 C]-Methylated molecular markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme) are shown in lane 1. The proteins were resolved by SDS/PAGE, processed for fluorography, and exposed on Kodak AR 5 X-ray film for 2 days.

Fig. 5. Synthesis of pI-6.4 esterase *in vitro* by RNA extracted from total, free and bound polysomes

Rat liver RNA extracted from total (lane 2), free (lane 3) and bound (lane 4) polysomes was translated in wheat-germ extract supplemented with [35 S]methionine (10 mCi/ml; 1200 Ci/mmol). Radiolabelled pI-6.4-esterase-related proteins were isolated by immunoprecipitation and analysed by SDS/PAGE and fluorography. Lane 1 was loaded with purified [3 H]di-isopropyl fluorophosphate-labelled pI-6.4 esterases.

readily bind with even small amounts of corresponding antibodies. The antiserum could be made monospecific by immunoadsorption on pI-6.1 esterase covalently linked to Sepharose-4B. The blots shown in Fig. 3 were probed with such an immunoaffinity-purified antiserum and 125 I-labelled protein A. Two bands were seen in the material focusing around pH 6.4, whether the proteins were resolved by non-denaturing, or SDS/PAGE (lanes 1 of panels *a* and *b* respectively). Freshly prepared Triton X-100 extracts showed a single immunoreactive peptide (lanes 2) that co-migrated with the slow band in the corresponding lane 1. A second fainter band, homologous with the slow-migrating component in lanes 1, was occasionally observed (results not shown). A single immunoreactive polypeptide corresponding to the band of apparent M_r 62000 in lane 1 of Fig. 3(*b*) was, however, consistently observed when crude microsomes were quickly boiled in SDS/PAGE sample buffer (lane 3). This strongly suggests that the polypeptide of M_r 60000 is generated during the purification procedure from the native enzyme of M_r 62000.

Molecular heterogeneity of the purified pI-6.4 esterases

To investigate whether the difference in electrophoretic mobility observed for the pI-6.4 esterases is due to a difference in glycosylation, a sample was subjected to digestion with endo-H, which cleaves high-mannose oligosaccharides and leaves one *N*-acetylglucosamine residue per oligosaccharide *N*-linked to the protein (Tarentino & Maley, 1974). At variance with the pI-5.0 esterases, which differ only in being glycosylated to variable extents (Robbi & Beaufay, 1986), the two pI-6.4

Fig. 6. Translocation and processing of the precursor form of pI-6.4 esterase by dog pancreatic microsomes

Total rat liver RNA was translated in rabbit reticulocyte lysate for 90 min at 26 °C in the absence (—) or presence (+) of EDTA-treated rough microsomes. Products from 50 μ l of translation were manipulated as specified, immunoprecipitated with anti-(pI-6.4 esterase) serum, analysed by SDS/PAGE and revealed by fluorography. Lane 3, translation in the absence of membranes; lanes 4 and 5, as lane 3, except that the translation products were diluted with unlabelled pI 6.4 (3 μ g) and pI-6.1 esterase (4 μ g) respectively before addition of the serum. In lanes 10 and 11 the material stems from the supernatants saved after immunoprecipitation with the anti-(pI-6.4 esterase) serum of samples 3 and 5 respectively and challenged with the anti-(pI-6.1 esterase) serum. Samples in lanes 6–8 derive from translation in the presence of EDTA-treated rough microsomes (2 A_{280} units/ml) and were analysed directly (6), or incubated with trypsin and chymotrypsin in the absence (7), or presence (8) of 1% Triton X-100. Lane 9, as in 3, except that the translation products were incubated post-translationally with microsomes and proteolysed with trypsin and chymotrypsin before immunoprecipitation. Lanes 1 and 2 show M_r markers and purified [3 H]di-isopropyl fluorophosphate-labelled pI-6.4 esterase respectively.

esterase forms behave as similarly glycosylated proteins differing slightly in polypeptide chain length (Fig. 4). Thus heterogeneity is probably the result of limited proteolysis, without inactivation of the enzyme.

Synthesis of the pI-6.4 esterase *in vitro*

Rat liver RNA extracted from total, free or bound polysomes, was translated in wheat-germ extract containing [35 S]methionine. Fig. 5 is a fluorogram of the immunoreactive translation products resolved by SDS/PAGE. A single product was identified when the system was programmed by RNA extracted from total (lane 2) or bound polysomes (lane 4); it was not formed when RNA extracted from free polysomes was used (lane 3). This product synthesized *in vitro* migrates half-way between the two [3 H]di-isopropyl fluorophosphate-labelled enzyme forms in the purified preparation (lane 1); its M_r is estimated at 61000.

Fig. 7. Affinity of the esterases synthesized *in vitro* for concanavalin A and sensitivity to endo-H

Translations were carried out in reticulocyte lysate in the absence (–) or in the presence (+) of dog pancreas rough microsomes (Micro) treated with EDTA ($2 A_{280}$ units/ml). In the latter case the products were incubated with trypsin and chymotrypsin to degrade the non-segregated material. (a): Lanes 1 and 2, immunoprecipitates of pI-6.4-esterase-related translation products analysed by SDS/PAGE and fluorography. Translation products of samples 3–5 were diluted with concanavalin A equilibration medium [10 mM-Tris/HCl (pH 7.5)/0.15 M-NaCl/1 mM-CaCl₂/1 mM-MnCl₂/1 % Triton X-100/5 mM-methionine/0.1 % serum albumin] containing no (lanes 3 and 4) or 0.2 M- α -methyl mannoside (α -MMan) (lane 5), and shaken for 3 h at room temperature with pre-equilibrated concanavalin A–Sepharose. The adsorbed products were removed by centrifugation. Immunoprecipitation was then carried out on the supernatants (lanes 3–5) and on the products eluted from the pellets with 2 % SDS at 100 °C, 5 min (lanes 6–8 respectively). (b) The immunoprecipitated products were incubated for 16 h at 37 °C in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of endo-H before SDS/PAGE.

Total rat liver RNA was also translated in reticulocyte lysate. The products displayed in Fig. 6 (lanes 3–5) are pI-6.4 esterase immunoprecipitates from similar translation products, but purified pI-6.4 and pI-6.1 esterase was added to samples 4 and 5 respectively before the antiserum. Again, a single 61 kDa polypeptide was recognized by the antiserum (lane 3); its precipitation was competed for by purified pI-6.4, but not by pI-6.1, esterase (lanes 4 and 5 respectively). Consequently the immunoprecipitated 61 kDa product is the pI-6.4 esterase precursor, and the antiserum no longer reacts with pI-6.1-esterase-related products. When dog pancreas microsomes were present in the cell-free translation system, the 61 kDa product was converted into a 62 kDa derivative (lane 6). This component was resistant to proteolytic degradation when the translation medium was incubated

Fig. 8. Biosynthetic labelling of pI-6.4 esterase

Cell monolayers of hepatocytes were preincubated for 1 h in methionine-free RPMI 1640 medium in the absence (–) or in the presence (+) of tunicamycin (TM) and subsequently labelled for 4 h with [³⁵S]methionine, still in the presence or in the absence of the antibiotic. Microsomal extracts (lanes 1 and 2, and 5–8) and culture media (lanes 3 and 4) were precipitated with the antiserum to pI-6.4 esterase. Immunoprecipitated products of lanes 5–8 were incubated for 16 h at 37 °C in the absence or in the presence of endo-H. Samples were analysed by SDS/PAGE and fluorography.

with trypsin and chymotrypsin in the absence of detergent (lane 7), but was fully degraded in the presence of Triton X-100 (lane 8), indicating that it was segregated in microsomal vesicles. Microsomes added after translation did not convert the precursor into the membrane-shielded form (lane 9). The supernatants of the pI-6.4-esterase immunoprecipitates analysed in lanes 3 and 5 were subjected to a second immunoprecipitation with anti-(pI-6.1 esterase) serum. The pI-6.1 esterase precursor was precipitated from sample 3 (lane 10), but less efficiently from sample 5, which was diluted with unlabelled pI-6.1 enzyme (lane 11). [It is noteworthy that pI-6.1 esterase causes limited proteolysis of its own primary translation product (compare lanes 10 and 11 in Fig. 6). Although proteinases are commonly assayed with esterase substrates, this is to our knowledge the first evidence that a carboxylesterase also shows proteolytic activity.] This definitely establishes the molecular identity of the pI-6.1 versus the pI-6.4 isoenzyme and the absence of cross-reactivity of the homologous antisera.

The pI-6.4 esterase synthesized in the presence of microsomes is identical with the mature enzyme; it binds to concanavalin A, except in the presence of α -methyl mannoside (Fig. 7a, lanes 4 and 5 respectively), and is sensitive to endo-H (Fig. 7b, lanes 3 and 4). As expected, the precursor showed no affinity for concanavalin A (a, lane 3), and was not sensitive to endo-H (b, lanes 1 and 2).

Biosynthesis of pI-6.4 esterase in primary cultured hepatocytes

Biosynthetic labelling with [^{35}S]methionine was carried out for 4 h in the absence or in the presence of tunicamycin, and immunoprecipitates from the cell extract and culture medium were analysed by SDS/PAGE and fluorography (Fig. 8). A single immunoreactive component was detected at 62 kDa in cell extracts of control hepatocytes (lane 1) and at 60 kDa in cells labelled in the presence of tunicamycin (lane 2). The 62 kDa component precipitated from control hepatocytes is sensitive to endo-H (lanes 5 and 6), in contrast with the 60 kDa form isolated from cells incubated with tunicamycin (lanes 7 and 8). The M_r difference between the glycosylated and the non-glycosylated, or deglycosylated forms confirms that the enzyme is *N*-glycosylated and endo-H-sensitive. The culture medium of control cells contained no immunodetectable pI-6.4 esterase (lane 3). Consistent with this, no serum protein reacts with the antiserum against pI-6.4 esterase (results not shown). That the culture medium of cells incubated in the presence of tunicamycin was also free of immunoprecipitable labelled material (lane 4) demonstrates that the *N*-linked oligosaccharide chain is not required for retention of pI-6.4 esterase in the cell.

Peptide mapping

The [^{35}S]methionine-labelled peptide fragments generated from the various pI-6.4 esterase forms (Fig. 9, lanes 1–5) are largely similar, but they differ from those generated from the pI 6.1 enzyme (lane 6). When generated from pI-6.4 esterase of hepatocytes, deglycosylated either by incubation of the enzyme with endo-H, or by biosynthesis in the presence of tunicamycin the peptide maps are indistinguishable (lanes 2 and 3). The map corresponding to the glycosylated enzyme (lane 1) shows both similarities with, and differences from, those of lanes 2 and 3. Indeed, glycosylation affects the electrophoretic mobility of the peptides and the sensitivity of the parent protein to proteolytic cleavage. The peptide map in lane 4, derived from the glycosylated protein synthesized under cell-free conditions, is similar to that in lane 1. Some difference in the enzyme/substrate ratio for the two samples probably accounts for the minor dissimilarities. The peptide maps of the precursor (lane 4) and processed product (lane 5) synthesized under cell-free conditions also reveal a high degree of homology. Apparently the *S. aureus* V8 proteinase readily cleaves the signal sequence, which is unique to the precursor, and glycosylation accounts for the slight differences between the two forms. It stems from these results that the immunoreactive peptides identified by our antiserum are truly related to the pI-6.4 esterase and in no way to the pI-6.1 enzyme (Robbi & Beaufay, 1987).

DISCUSSION

Preparations of pI-6.4 esterase obtained, by necessity, in the absence of proteinase inhibitors, contain two peptides, reacting with [^3H]di-isopropyl fluorophosphate, of slightly different M_r value (Fig. 1). Immunotitration (Fig. 2) demonstrates that the pI-6.4 esterase forms present in crude extracts are immunologically distinct from the other major rat liver carboxylesterases. The antiserum still cross-reacted with pI-6.1 esterase on

Fig. 9. Peptide maps of pI-6.4 esterases labelled *in vivo* and *in vitro*.

Radioactive peptides were excised from lanes 5–7 of Fig. 8, and lanes 2 and 1 of Fig. 7, electrophoretically eluted and digested for 30 min at room temperature with 1 μg of *S. aureus* V8 proteinase as described in the Experimental section. The resulting peptide fragments were analysed on a 15%-(w/v)-polyacrylamide/SDS gel and the ^{35}S -labelled ones were revealed by fluorography (lanes 1–5). The peptide fragments displayed in lane 6 were obtained from similarly processed pI-6.1 esterase excised from Fig. 6, lane 10. Abbreviations: RER, rough endoplasmic reticulum; TM, tunicamycin.

Western blots. Because the latter enzyme is a homotrimer (Mentlein *et al.*, 1980; Robbi & Beaufay, 1983) migrating much more slowly in non-denaturing gels than the monomeric pI-6.4 esterase forms, we suspect that a trace of monomeric pI-6.1 esterase is present in the antigen. Hence, it might be virtually impossible to produce directly a specific antiserum against the pI-6.4 esterase, but contaminating antibodies could be removed by adsorption on pI-6.1 esterase bound to Sepharose-4B.

The polymorphism displayed by pI-6.4 esterase is 'artefactually' generated by limited proteolysis of the authentic enzyme (see Fig. 3). In agreement with this interpretation, the antiserum detects a single molecular species in biosynthetically labelled products. Others identified two rat liver esterases focusing at pH 6.2 and 6.4, and reported their high degree of immunochemical and functional relatedness (Mentlein *et al.*, 1984a,b). As pI values determined in different laboratories may differ slightly, these esterases most likely correspond to the pI-6.4 esterase forms observed here.

The biosynthesis of pI-6.4 esterase has been faithfully reproduced in two cell-free systems and in cultured hepatocytes. The protein is synthesized on bound polysomes as a precursor polypeptide which is co-translationally processed by removal of a short peptide (≈ 1 kDa) and concomitant *N*-glycosylation. Thus it cannot be distinguished from proteins that follow the

secretory pathway. The non-glycosylated protein synthesized in hepatocytes poisoned with tunicamycin is retained in the cells like the glycosylated enzyme. Therefore glycosylation does not label the molecule with the structural determinant responsible for retention in the ER.

Munro & Pelham (1987) reported that the C-terminal sequence -Lys-Asp-Glu-Leu, common to three luminal proteins of the ER, namely grp 78 (BIP), grp 94 and protein disulphide-isomerase, is required for their retention in ER and prevents the exit of an engineered derivative of lysozyme. According to Mentlein *et al.* (1984a), the C-terminal sequences of pI-6.4 and -6.1 esterases are -Glu-Val-Xaa (Xaa = Ser, Asn or Gln) and -Ala-Val-Leu respectively. If these are the C-terminal ends of the authentic unaltered molecules, another sorting mechanism must be responsible for their retention in the ER. Other proteins are retained, at least transiently, through a different mechanism. The microsomal β -glucuronidase interacts with the catalytic centre of esterase 3, another carboxylesterase, which in this case acts as an anchor protein (Medda *et al.*, 1987a,b). How esterase 3 itself is retained in the ER is not known. Heavy chains of immunoglobulins bind transiently to luminal protein BIP, which delays their delivery from ER until they are adequately associated with light chains (Bole *et al.*, 1986).

As the carboxylesterases of the ER share a number of physico-chemical properties, they are probably sorted out of the secretory traffic by the same mechanism, probably one involving a common structural feature which remains to be identified.

We are grateful to Dr. Philippe Pirson (International Institute of Cellular and Molecular Pathology, Brussels) for kindly providing the cultured hepatocytes. This research was supported by the Belgian 'Fonds de la Recherche Fondamentale Collective (n° 2.4540.84)' and by the Belgian State - Prime Minister's Office - Science Policy Programming (inter-university attraction poles, grant n°. 7, and concerted actions, grant n°. 82/87-39).

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Received 30 October 1987/4 March 1988; accepted 9 May 1988