Characterization of trimming Man₉-mannosidase from pig liver

Purification of a catalytically active fragment and evidence for the transmembrane nature of the intact 65 kDa enzyme

Jürgen SCHWEDEN and Ernst BAUSE*

Institut für Biochemie, Zùlpicherstrasse 47, 5000 Köln 1, Federal Republic of Germany

An α 1,2-mannosidase (Man₉-mannosidase) involved in *N*-linked oligosaccharide processing has been purified about 16000-fold from pig liver crude microsomes (microsomal fractions) by CM-Sepharose and DEAE-Sephacel chromatography, concanavalin A (Con A)-Sepharose chromatography and, as the key step of the procedure, affinity chromatography on immobilized *N*-5-carboxypentyl-1-deoxymannojirimycin (CP-dMM). On SDS/polyacrylamide-gel electrophoresis under reducing conditions, the isolated enzyme migrated as a single protein band with a molecular mass of 49 kDa. The enzyme does not bind Con A and is not susceptible to glycopeptidase F, indicating that it lacks *N*-linked oligosaccharides of the high-mannose or complex type. Purified Man₉-mannosidase has a pH optimum close to 6.0 and requires bivalent cations for activity, with Ca²⁺ being most effective. The enzyme is inhibited strongly by basic sugar analogues of mannose such as 1-deoxymannojirimycin (dMM, $K_i \approx 5 \mu\text{M}$), *N*-methyl-dMM ($K_i \approx 55 \mu\text{M}$) and CP-dMM ($K_i \approx 150 \mu\text{M}$), whereas *NN*-dimethyl-dMM and the mannosidase II inhibitor swainsonine were hardly or not at all inhibitory. A homogeneous preparation of the 49 kDa enzyme cleaves specifically three of the four α 1,2-mannosidic linkages in the natural Man₉-GlcNAc₂ (M₉) substrate. The relative rates by which the parent and intermediate structures are hydrolysed were found to be about 3:2:5 for M₉, M₈ and M₇, respectively. The enzyme displays only marginal activity toward the remaining α 1,2-mannosidic linkages in the Man₆-GlcNAc₂ oligosaccharide (relative rate of M₆ hydrolysis ≈ 0.02) and is not active against nitrophenyl and methylumbelliferyl α -mannosides. This unique substrate specificity suggests that Man₉-mannosidase processing differs from that catalysed by other trimming α 1,2-mannosidases hitherto reported. A polyclonal antibody raised against the denatured 49 kDa polypeptide not only recognizes a protein band of similar size in Western blots of crude microsomes, but also reacts strongly with a 65 kDa protein species. On trypsin treatment of detergent-solubilized microsomes, the 65 kDa protein is converted specifically into a stable 49 kDa fragment, indicating a precursor-product relationship between the two proteins. We conclude from this observation that the 65 kDa protein represents the intact form of Man₉-mannosidase from which the 49 kDa enzyme which we have isolated has been generated, with retention of catalytic activity, by proteolysis during purification. Proteolytic studies with sealed microsomes suggest that the intact 65 kDa enzyme is a protein with a membrane-spanning domain, as well as a cytosolic polypeptide domain of size at least 3 kDa.

INTRODUCTION

The major pathway of *N*-glycan formation involves transfer of a common Glc₃-Man₉-GlcNAc₂ precursor from a lipid intermediate to protein, followed by oligosaccharide processing [1]. Processing begins with the removal of the three glucose residues by glucosidases I and II. Up to four α 1,2-linked mannose residues are then cleaved by specific endoplasmic-reticulum (ER) and/or Golgi α -mannosidases, resulting in the formation of different types of 'high-mannose' oligosaccharide. The key step in the conversion of a 'high-mannose' into a 'complex'-type structure, is the addition of a single *N*-acetylglucosamine (GlcNAc) residue to the Man₅-GlcNAc₂ heptasaccharide core catalysed by *N*-acetylglucosaminyl-

transferase I. The GlcNAc-Man₅-GlcNAc₂ hybrid which results is then acted upon by mannosidase II, which by cleaving the unblocked α 1,3-/ α 1,6-mannosyl branch, gives rise to a GlcNAc-Man₃-GlcNAc₂ intermediate. This intermediate is modified further to yield 'complex'-type glycans by subsequent transfer of additional GlcNAc, galactose, *N*-acetylneuraminic acid and fucose residues.

Recently several α -mannosidases, obtained from different tissues, have been characterized which specifically cleave α 1,2-mannosidic linkages in the Man₉-GlcNAc₂ oligosaccharide. Although similar in some respects, these α 1,2-mannosidases differ in a number of other properties, most notably in their substrate specificity and subcellular location. In intact hepatocytes

Abbreviations used: (CP-)dMM, (*N*-5-carboxypentyl)-1-deoxymannojirimycin; Nph-, nitrophenyl-; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; Con A, concanavalin A; ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; Endo H, endo- β -*N*-acetylglucosaminidase H; AH-, aminohexyl; PBS, phosphate-buffered saline (composition given in the text.)

* To whom correspondence and reprint requests should be sent.

an ER α -mannosidase has been identified which seems to preferentially remove one specific mannose residue from protein-bound $\text{Man}_9\text{-GlcNAc}_2$ structures to form a single $\text{Man}_8\text{-GlcNAc}_2$ isomer [2]. We have purified an $\alpha 1,2$ -mannosidase (Man_9 -mannosidase) from calf liver which, at least under the conditions used *in vitro*, is capable of removing three of the four $\alpha 1,2$ -mannose residues from the $\text{Man}_9\text{-GlcNAc}_2$ substrate and which we have postulated as being an ER-resident enzyme [3]. Recently, a rabbit liver $\alpha 1,2$ -mannosidase has been reported by Forsee *et al.* [4], which shares a number of physical and enzymic properties in common with Man_9 -mannosidase from calf liver, suggesting that both enzymes fulfil the same function in the oligosaccharide-processing sequence. In addition, two Golgi-associated $\alpha 1,2$ -mannosidases (mannosidase IA/IB) have been identified and (partially) purified which cleave all four $\alpha 1,2$ -mannosidic linkages in Man_9 -oligosaccharides with similar efficiency, thereby providing the key substrate for the pathway of complex-type sugar formation [5,6]. The biological significance of the observed multiplicity of trimming $\alpha 1,2$ -mannosidases, as well as the molecular signals operating in their regulation, are poorly understood. It appears likely, however, that these enzymes catalyse alternative processing routes by which distinct groups of *N*-glycoproteins may be modified.

Here we report the purification and characterization of an $\alpha 1,2$ -mannosidase (Man_9 -mannosidase) from pig liver which cleaves specifically $\text{Man}_9\text{-GlcNAc}_2$ to $\text{Man}_8\text{-GlcNAc}_2$ oligosaccharides. The overall properties of the pig liver enzyme, including its unique substrate specificity, closely resemble those of a Man_9 -mannosidase recently isolated from calf liver [3]. A polyclonal antibody raised against the purified 49 kDa enzyme protein was found to bind, in addition to the 49 kDa antigen, to a prominent band at 65 kDa in Western blots of crude microsomes (microsomal fractions). Several lines of evidence suggest that this 65 kDa protein is a transmembrane protein and represents the intact form of Man_9 -mannosidase, which is converted into the catalytically active 49 kDa fragment by proteolysis.

MATERIALS AND METHODS

Materials

Chemicals and compounds were supplied from the following sources: GDP-[^{14}C]mannose (sp. radioactivity 306 Ci/mol), Amersham International; goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, Nitro Blue Tetrazolium chloride, nucleotide sugars, detergents, molecular-mass standards, Sigma (Taufkirchen); pepstatin, leupeptin, PMSF, Fluka; nitrophenyl (Nph) α -mannoside, endo- β -*N*-acetylglucosaminidase H (Endo H), glycopeptidase F, Boehringer; Bio-Gel P4 (< 400 mesh), Bio-Rad Laboratories; Sephadex G-25, DEAE-Sephacel, aminohexyl (AH)-Sephacel, CM-Sephacel, concanavalin A (Con A)-Sephacel, protein A-Sephacel, Pharmacia. Pig liver was obtained from the local slaughterhouse. All other chemicals were of the highest purity available from commercial sources.

Methods

Buffers: (A) 250 mM-sucrose in 10 mM-phosphate, pH 6.5; (B) 10 mM-phosphate, pH 6.5; (C) 200 mM-phosphate, pH 6.0, containing 1% Lubrol PX; (D)

45 mM-phosphate, pH 5.9, containing 1% Lubrol PX; (E) 50 mM-phosphate, pH 6.5, containing 1% Lubrol PX; (F) 100 mM-phosphate, pH 7.5; (G) 0.15 M-NaCl in 10 mM-phosphate, pH 7.2 (phosphate-buffered saline, PBS). If not otherwise stated, buffers A–E contained 10 μM -PMSF and 1 mM-2-mercaptoethanol.

Preparation of pig liver crude microsomes. Pig liver (10 kg) was freed of connective tissue, suspended in 4 vol. of buffer A and minced in a Waring blender. The suspension was homogenized in a Potter–Elvehjem homogenizer (clearance 0.095–0.115 mm) by using four or five up-and-down strokes of a pestle revolving at 1500 rev./min. The homogenate was centrifuged at 14000 *g* for 30 min and the supernatant retained. The pellet was resuspended in 2 vol. of buffer A, homogenized and centrifuged as described above. The 14000 *g* supernatants were combined and CaCl_2 was added (final concn. 10 mM), followed by centrifugation at 48000 *g* for 120 min to yield the crude microsomal fraction. The crude microsomes were washed by resuspending the membrane pellet in buffer B, followed by centrifugation as described above. For Western-blot analyses microsomes were prepared, where stated, in the presence of a proteinase-inhibitor cocktail containing pepstatin (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$) and PMSF (10 μM).

Purification of Man_9 -mannosidase. Man_9 -mannosidase activity was solubilized by suspending washed pig liver crude microsomes (about 1000 ml of membrane pellet) in 10 vol. of buffer C at a final protein concentration of ≈ 10 mg/ml. The suspension was kept on ice for 30 min, diluted with 4 vol. of ice-cold 1% Lubrol PX in water to decrease the sodium phosphate concentration to about 45 mM and the pH adjusted to 5.9 with HCl. CM-Sephacel, equilibrated in buffer D, was added to the diluted extract, and the suspension was stirred for 5 h. The ion-exchange matrix was separated by filtration, washed several times with buffer D, and bound mannosidase activity was eluted with buffer C. For affinity binding of Man_9 -mannosidase, 2 ml of the CP-dMM-AH-Sephacel 4B affinity resin (ligand concentration 3–5 $\mu\text{mol}/\text{ml}$ of gel) were added to the CM-Sephacel eluate and the suspension stirred for 12 h. The affinity matrix was separated by decantation, washed thoroughly with buffer C, and Man_9 -mannosidase was eluted batchwise with 10 mM-dMM in buffer C. The inhibitor was removed by passing the affinity eluate over a Sephadex G-25 column (1.5 cm $\times$ 75 cm), previously equilibrated in buffer E. The affinity-purified material was then treated batchwise, and in series, with 1 ml each of Con A-Sephacel and DEAE-Sephacel, both matrices being equilibrated in buffer E. The latter step removed traces of Con A, which were introduced by the lectin-binding step. The unbound fraction, containing Man_9 -mannosidase, was collected and the enzyme was concentrated by binding to, and elution from, CM-Sephacel under the conditions described above for the initial purification step. All operations were carried out at 4 $^\circ\text{C}$.

Preparation and purification of a polyclonal anti-(Man_9 -mannosidase) antibody. SDS-denatured pig liver Man_9 -mannosidase (200 μg) in 0.5 ml of buffer C was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into a rabbit. Immunization was repeated with 100 μg of the enzyme in incomplete

Freund's adjuvant after 4 and 8 weeks. The blood obtained 2 weeks after the last booster injection was collected, allowed to clot at room temperature, and centrifuged at low speed to yield the antiserum.

The IgG fraction was isolated from the antiserum by passing it over a protein A-Sepharose column (2 ml), which was equilibrated in buffer F. After several washes with buffer F, the IgG fraction was eluted with 0.1 M-glycine, pH 3.0. To avoid antibody denaturation, the eluate was immediately neutralized by the addition of 1 M-Tris buffer, pH 8.0. The anti-(Man₆-mannosidase) antibody was separated from the IgG fraction by affinity chromatography on a column (2 ml) containing the antigen covalently attached to Sepharose 4B ($\approx 100 \mu\text{g}/\text{ml}$ of gel). Synthesis of the affinity resin followed the protocol described by Pharmacia for the coupling of proteins to CNBr-activated Sepharose 4B. After several washes of the affinity matrix with PBS, the antigen-antibody complex was dissociated with 0.1 M-glycine, pH 2.8, followed by neutralization of the acidic eluate. As determined by e.l.i.s.a., this two-step purification increased the anti-(Man₆-mannosidase) antibody titre about 250-fold over that of the crude antiserum. After dialysis against PBS, the purified antibody was stored at -20°C .

Trypsin treatment of intact and detergent-solubilized microsomes. Freshly prepared microsomes were incubated at 37°C in 100 mM-phosphate buffer, pH 7.5, with trypsin at a proteinase/protein ratio of 1:100 in the absence or presence of 0.3% Triton X-100. After the times indicated, the samples were diluted with an appropriate volume of Laemmli sample buffer [7] and the proteins immediately denatured by heating to 100°C for 5 min; the cleavage products were then analysed by SDS/PAGE and immunoblotting.

SDS/PAGE and immunoblotting. Proteins were analysed by SDS/PAGE according to the procedure originally described by Laemmli [7] and stained with either Coomassie Blue R-250 (polyacrylamide gels) or Indian Ink (after blotting on to nitrocellulose). Immunoblots were obtained after electrophoretic transfer of the proteins on to nitrocellulose [8] and reaction of the replica with anti-(Man₆-mannosidase) antibody, followed by identification of antigen-antibody complexes with a goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue tetrazolium chloride as substrates.

Glycosidase assays. Man₆-mannosidase activity was measured with [^{14}C]Man₆-GlcNAc₂ as described previously [3]. One enzyme unit is defined as the amount of enzyme which catalyses the release of 1% [^{14}C]mannose/min from the radiolabelled oligosaccharide at 37°C . Non-specific α -mannosidase activity was determined with 5 mM-Nph α -mannoside, and released nitrophenol was measured spectrophotometrically [9]. One Nph- α -mannosidase unit corresponds to the amount of enzyme hydrolysing $1 \mu\text{mol}$ of Nph α -mannoside/min at 37°C .

The pH-dependence of purified Man₆-mannosidase was determined in the presence of either 0.1 M-acetate (pH 4.5–5.5) or 0.1 M-phosphate (pH 5.5–7.5) buffer. Inhibition studies were carried out by incubating the

purified enzyme with various concentrations of inhibitor(s) at 4°C for 10 min and determining residual enzyme activity with [^{14}C]Man₆-GlcNAc₂ at 37°C .

General procedures. The synthesis and purification of [^{14}C]Man₆-GlcNAc₂ and the analysis by h.p.l.c. of radiolabelled oligosaccharides were as previously described [3]. dMM, CP-dMM and the CP-dMM-AH-Sepharose 4B affinity resin were synthesized as previously reported [10,11]. Protein was determined by the method of Lowry *et al.* [12], with bovine serum albumin as the standard. E.l.i.s.a was performed using standard procedures [13]. Mannose-6-phosphatase activity was assayed as described in [14]. Digestion with glycopeptidase F was performed as described in the supplier's manual. Staining of sugars was performed by the dansyl-hydrazine method [15]. Radioactivity was determined in a liquid-scintillation counter (Delta 300, Searle Analytic) using Bray's solution as counting fluid [16].

RESULTS AND DISCUSSION

Purification of trimming Man₆-mannosidase from pig liver

Crude liver microsomes contain a variety of α -mannosidase activities, which can be distinguished, at least partially, by differences in their substrate specificity, pH-dependence, detergent requirements and inhibition by various mannosidase inhibitors [1,3]. We have focused on the isolation from pig liver of one particular processing α 1,2-mannosidase (Man₆-mannosidase), which cleaves Man₆-GlcNAc₂ oligosaccharides and which is strongly inhibited by the basic sugar analogue dMM and its *N*-alkyl derivatives. The inhibition by *N*-alkylated dMM derivatives formed the basis for the design and synthesis of an affinity ligand, the structure of which is shown in Fig. 1. The protocol for the purification is outlined in Scheme 1. The efficiency was monitored by measuring

Scheme 1. Outline of the purification procedure

Fig. 1. Structure of CP-dMM

Table 1. Summary of a typical Man₉-mannosidase purification

Man₉-mannosidase activity was assayed with [¹⁴C]Man₉-GlcNAc₂; 'non-specific' aryl- α -mannosidases were assayed with Nph α -mannoside as substrate. For definition of units, see the Materials and methods section. Abbreviation: n.d., not detectable under standard assay conditions.

Fraction	Volume (ml)	Protein (mg)	Aryl- α -mannosidase		Man ₉ -mannosidase			
			Activity (munits)	Sp. activity (munits/mg)	Activity (units)	Sp. activity (units/mg)	Purification (fold)	Yield (%)
Pig liver crude microsomes, salt/detergent extract	26000	104000	37000	0.36	84600	0.81	1	100
CM-Sepharose eluate	312	2900	1600	0.57	48500	16.7	20.6	57
Affinity eluate	32	4.8	1.25	0.26	22200	4610	5700	26
Con A-Sepharose, unbound fraction	34	4.0	n.d.	—	20650	5100	6300	24
Purified Man ₉ -mannosidase (after DEAE-Sephacel treatment and concentration on CM-Sepharose)	3.0	1.18	n.d.	—	15000	12700	15700	19

the enzyme activity with [¹⁴C]Man₉-GlcNAc₂ and *p*-Nph α -mannoside as the substrates at each step of the procedure.

The results of a typical isolation starting from 10 kg of pig liver is summarized in Table 1. Quantitative solubilization of α 1,2-mannosidase activity from the microsomal fraction was found to require high concentrations of salt and detergent (buffer C); these rather drastic conditions were, however, not essential at later stages of the purification to keep the enzyme in solution. After dilution, the high-salt/high-detergent extract was treated with CM-Sepharose. About 60 % of the [¹⁴C]Man₉-GlcNAc₂-hydrolysing activity bound to the ion-exchange resin, whereas > 95 % of the non-specific aryl- α -mannosidase activity remained in the supernatant. The Man₉-GlcNAc₂-hydrolysing activity in the unbound fraction neither bound to newly equilibrated CM-Sepharose, nor did it contain protein species cross-reacting with the antibody against the purified (49 kDa) Man₉-mannosidase. These observations point to the presence of distinct α 1,2-mannosidases in the detergent extract which have different ion-exchange-resin-binding properties. The CM-Sepharose-adsorbed mannosidase activity was eluted with buffer C, yielding an enzyme preparation with a 21-fold increase in specific activity for the Man₉-oligosaccharide hydrolase(s), compared with only 1.6-fold for the aryl- α -mannosidases.

Affinity chromatography of the CM-Sepharose eluate on CP-dMM-AH-Sepharose 4B (Fig. 1) was performed by adding the affinity matrix batchwise to the eluate. Between 60–80 % of the [¹⁴C]Man₉-GlcNAc₂-hydrolysing activity became adsorbed to the affinity matrix after 12 h, whereas the bulk of aryl- α -mannosidase(s) remained in the supernatant. This binding pattern was not altered by changes in the reaction time, pH or amount of affinity gel added, indicating that the seemingly incomplete binding of Man₉-GlcNAc₂-hydrolysing activity must be due to the presence of enzyme(s) differing

in their immobilized-ligand-binding properties. We did not characterize the unbound fraction further. The affinity-bound Man₉-mannosidase activity was eluted with dMM, followed by column chromatography on Sephadex G-25 to separate enzyme and inhibitor. The amount of Man₉-mannosidase recovered in the eluate was 30–50 % of the activity initially bound to the affinity resin. The affinity step increased the specific activity of Man₉-mannosidase by 280-fold with an overall purification of 5700-fold over crude microsomes. SDS/PAGE of the affinity-purified material (Fig. 2, lane 3), and trace amounts of Nph- α -mannosidase activity in the affinity eluate, indicated that the Man₉-mannosidase preparation was not homogeneous at this stage.

Further purification was achieved by treating the affinity-purified enzyme with Con A-Sepharose and DEAE-Sephacel. Con A-Sepharose was found to remove any residual Nph- α -mannosidase activity which bound to the immobilized lectin. Final treatment of the Man₉-mannosidase preparation with DEAE-Sephacel removed remaining protein and Con A contaminant(s), the latter resulting from 'bleeding' of the lectin. The unbound Man₉-mannosidase was then concentrated with CM-Sepharose as described above.

The final Man₉-mannosidase preparation was greatly (about 16000-fold) enriched compared with crude microsomes. SDS/PAGE under reducing conditions revealed a single protein band at 49 kDa, indicating the homogeneity of the enzyme preparation (Fig. 2, lane 4). In several purifications the overall recovery of Man₉-mannosidase was between 15 and 20 %, on the basis of the Man₉-GlcNAc₂-hydrolysing activity in the microsomes. It should be noted, however, that both purification factor and enzyme recovery are likely to be higher, since their calculation did not take account of the presence of other α -mannosidases in the microsomal preparation which also act on the [¹⁴C]Man₉-GlcNAc₂ oligosaccharide.

Fig. 2. SDS/PAGE of pig liver Man₉-mannosidase at various stages in its purification

Enzyme samples obtained after various stages of the purification were subjected to SDS/PAGE as described by Laemmli [7] and the gel stained with Coomassie Blue. Lane 1, pig liver crude microsomes; lane 2, CM-Sepharose eluate; lane 3, eluate after affinity chromatography; lane 4, purified pig liver Man₉-mannosidase; lane 5, molecular-mass standards.

Molecular and enzymic properties of pig liver Man₉-mannosidase

Under reducing and denaturing conditions, Man₉-mannosidase migrated on SDS/PAGE as a single protein band with a molecular mass of 49 kDa (Fig. 2, lane 4). The enzyme activity did not bind to Con A-Sepharose, indicating that the 49 kDa protein does not contain high-mannose oligosaccharide chain(s). This view is supported by the observation that neither the native nor the denatured 49 kDa enzyme was susceptible to cleavage by glycopeptidase F. In addition, no sugar staining was detectable (after SDS/PAGE) by the dansylhydrazine method [15] under conditions where comparable amounts of ovalbumin, an *N*-glycoprotein with one sugar chain of presumably similar size, gave unambiguous results (result not shown). We assume, therefore, that the 49 kDa protein is not glycosylated.

The pH optimum of Man₉-mannosidase is close to pH 6.0, with half-maximal activity at pH 5.2 and 6.7 respectively. The rather steep decrease of activity towards the acidic site clearly discriminates this trimming enzyme from lysosomal hydrolases.

[¹⁴C]Man₉-GlcNAc₂ hydrolysis by the purified 49 kDa enzyme is strongly inhibited by EDTA and other bivalent-metal-ion chelators. Full enzymic activity is recovered, however, when the incubation mixtures are supplemented with millimolar concentrations of certain bivalent cations: for re-activation, Ca²⁺ ions turned out to be more effective than either Zn²⁺ or Mg²⁺, whereas Cu²⁺

ions were found to be inhibitory. A similar requirement for bivalent-metal ions was recently observed for the Man₉-mannosidase from calf liver [3] and other trimming α -mannosidases [4,5,17]; most α -glucosidases (including trimming glucosidase I [18,19]), however, appear not to be metal-ion-dependent. The general need for metal ions in the catalytic mechanism of α -mannosidases may reflect some peculiarities not present in the case of glucosidases. These differences may originate from structural constraints imposed by the axial configuration of the C-2 hydroxy group in the mannose-containing substrate.

The substrate specificity of the pig liver enzyme is identical with that recently determined for Man₉-mannosidase from calf liver [3]. Both enzymes exclusively accept 'high-mannose' oligosaccharides as substrates, but are not active towards synthetic aryl or umbelliferyl α -mannosides. A typical time course of [¹⁴C]Man₉-GlcNAc₂ hydrolysis by the purified pig liver enzyme is presented in Fig. 3. Fig. 3(a) shows the h.p.l.c. patterns of cleavage products obtained after different incubation times, from which the kinetics in Fig. 3(b) are derived. The data are corrected on the assumption that the radiolabel in the [¹⁴C]oligosaccharide is evenly distributed between all the mannose residues. Pig liver Man₉-mannosidase is seen to cleave readily three of the four α 1,2-mannosidic linkages in the Man₉-substrate. The enzyme displays only marginal activity toward the [¹⁴C]Man₆-GlcNAc₂ intermediate, which, as a consequence, accumulates. Even after extended incubation times (resulting in more than 90% conversion of [¹⁴C]Man₉-GlcNAc₂ into [¹⁴C]Man₆-GlcNAc₂), less than 5% of the radioactivity is found in a product eluted on h.p.l.c. in the position of [¹⁴C]Man₅-GlcNAc₂. The molecular reason for the unusual resistance of the α 1,2-mannosidic linkage, still present in [¹⁴C]Man₆-GlcNAc₂, is not understood. From the kinetics in Fig. 3(b), it can be estimated that the relative rates for the conversion of Man_x-GlcNAc₂ to the next lower substrate, are about 1.5 ($x = 9$):1 ($x = 8$):2.5 ($x = 7$):0.02 ($x = 6$). A similar ratio was recently determined for calf liver Man₉-mannosidase ($M_9:M_8:M_7 = 1.8:1:3.5$), suggesting that both enzymes catalyse the same steps in the *N*-linked processing pathway. Further evidence for the functional identity of pig and calf liver Man₉-mannosidase is provided by the observation that the K_i values for their inhibition by dMM and several of its *N*-alkyl derivatives are comparable. These data, as well as some other enzymic and physical properties of purified pig liver Man₉-mannosidase, are summarized in Table 2; in order to demonstrate the close relationship between the two enzymes, data for the calf liver enzyme are also included.

Characterization of a polyclonal antibody against the SDS-denatured 49 kDa enzyme

A polyclonal antibody was prepared in a rabbit against the denatured 49 kDa enzyme in order to have a specific analytical probe available for further studies (for details, see the Materials and methods section). The affinity-purified antibody was found to bind to the denatured 49 kDa enzyme with high affinity on Western blots. As little as 1–2 ng of Man₉-mannosidase were detected easily by using an alkaline phosphatase-conjugated second antibody for staining. Non-specific staining did not occur with the pre-immune serum at concentrations even higher than those which gave strong positive signals with the purified antibody (results not shown). It appears, there-

Fig. 3. Substrate specificity of pig liver Man₉-mannosidase

Purified Man₉-mannosidase was incubated with [¹⁴C]Man₉-GlcNAc₂ under standard assay conditions. At given times, aliquots were withdrawn from the incubation, the samples briefly heated to denature the enzyme and the products analysed by h.p.l.c. on a Nucleosil NH₂ column, using an acetonitrile/water gradient for elution as described in [3]. (a) H.p.l.c. fractionation pattern of cleavage products; (b) [¹⁴C]oligosaccharide hydrolysis as a function of time. The values in both (a) and (b) are corrected on the assumption that the radiolabel in the parent [¹⁴C]Man₉-GlcNAc₂ oligosaccharide is evenly distributed. Abbreviations and symbols: M₉₋₅, [¹⁴C]Man₉₋₅-GlcNAc₂; ●, M₉; ○, M₈; △, M₇; ▲, M₆; ×, M₅.

fore, that the anti-(Man₉-mannosidase) antibody is quite specific for the 49 kDa protein.

For further characterization, the crude serum and the affinity-purified antibody were tested for their ability to bind native Man₉-mannosidase. Surprisingly, and in contrast with the high affinity to the SDS-denatured enzyme on Western blots, both the antiserum and the purified antibody failed to immunoprecipitate native Man₉-mannosidase. This finding suggests that the antibody prepared against the denatured 49 kDa enzyme

does not contain species capable of interacting with structural epitopes on the surface of the native enzyme. An antibody recently raised against the native 49 kDa enzyme was found to immunoprecipitate Man₉-mannosidase activity with high efficiency and also to bind to the denatured enzyme protein on Western blots (E. Bause & J. Schweden, unpublished work). For the experiments described below, we have exclusively used the antibody against the denatured (49 kDa) Man₉-mannosidase as an analytical tool.

Table 2. Molecular and enzymic properties of purified Man₉-mannosidase from pig and calf liver

Property	Man ₉ -mannosidase from:	
	Pig liver	Calf liver†
Molecular mass (SDS/PAGE, reducing conditions)	49 kDa	56 kDa
Con A-Sepharose binding	None	None
Metal-ion-dependent?	Yes	Yes
pH optimum	6.0	6.0–6.2
Substrate specificity:		
[¹⁴ C]Man ₉ -GlcNAc ₂	Removal of three α 1,2-linked mannoses	
[¹⁴ C]Man ₉ -GlcNAc ₂ Nph α -mannosides	No hydrolysis	
Inhibition* by:		
dMM	$K_i \approx 5 \mu\text{M}$	$K_i \approx 7 \mu\text{M}$
N-Methyl-dMM	$K_i \approx 55 \mu\text{M}$	$K_i \approx 75 \mu\text{M}$
CP-dMM	$K_i \approx 150 \mu\text{M}$	$K_i \approx 140 \mu\text{M}$
NN-Dimethyl-dMM	> 10% Inhibition by 0.5 mM	Not determined
Swainsonine	No inhibition by 1.0 mM	Not determined

* Determined at the pH optimum of activity.

† Data taken from [3].

Intact Man₉-mannosidase has a molecular mass of 65 kDa

When detergent-solubilized extracts of pig liver crude microsomes were subjected to SDS/PAGE followed by immunoblotting, the affinity-purified antibody recognized, in addition to the 49 kDa enzyme protein, several smaller polypeptides as well as two larger protein species (of size 62 and 65 kDa). None of these protein bands were stained using the pre-immune serum for detection, indicating that they were Man₉-mannosidase-specific. The complexity of the pattern and the ratio of band intensities changed drastically, depending on the length of time for which the detergent extracts were stored, and also on whether proteinase inhibitors were present during their preparation (results not shown). This suggested that the observed changes in the immunoreactivity pattern were generated by non-specific proteolysis of a parent protein.

In order to demonstrate the presumed product-precursor relationship between the immunostainable proteins, freshly prepared microsomes were incubated for various times with trypsin in the presence of 0.3% Triton X-100 and the cleavage products were analysed immunoblotting. The results of a typical time-course experiment is presented in Fig. 4(a). It is evident from lane 2 (zero-time point, control) that the 65 kDa protein is the prominent immunostainable species in crude microsomes immediately after the addition of detergent and proteinase. The concentration of the 65 kDa protein decreases rapidly with time, however, indicating that it is highly susceptible to proteolytic cleavage; concomitant with its degradation, a rather stable protein of 49 kDa appears that is the main product even after as little as 2 min of proteolysis. Since no intermediate products accumulate, we conclude that the 49 kDa protein arises

directly from the 65 kDa protein. This would imply that the 65 kDa protein, and not the 49 kDa species, represents the intact (and previously membrane-associated) form of Man₉-mannosidase. This interpretation is supported by the observation that the antibody failed to cross-react with protein species larger than 65 kDa in crude microsomes.

Pig liver Man₉-mannosidase is a transmembrane protein with a cytosolic domain

In order to study the membrane association and topology of Man₉-mannosidase in more detail, proteolysis experiments were carried out with freshly prepared microsomes in the absence of detergents (to maintain vesicle integrity). The integrity of the microsomes was demonstrated by a pronounced latency of glucosidase I and mannose-6-phosphatase activity, which was lost on treatment with Triton X-100, manifested by a several-fold increase in Glc₃-Man₉-GlcNAc₂ and mannose-6-phosphate hydrolysis [11,14]. The crypticity of both activities also indicates that the microsomal vesicles must have the same membrane topology as that found *in situ*. Fig. 4(b) shows that the immunoblot pattern obtained in the absence of detergent differs markedly from that obtained with solubilized microsomes. Under the conditions which prevent trypsin gaining access to the luminal space, the 65 kDa protein is converted specifically into a 62 kDa species. No smaller cleavage products are seen, even after longer incubation times, indicating that the 62 kDa fragment is protected against further degradation. We conclude from this observation: (i) that the intact 65 kDa form of Man₉-mannosidase is a transmembrane protein, and (ii) that it bears a cytoplasmically located polypeptide domain of size at least 3 kDa.

Conclusions

The five-step procedure described in the present paper for the purification of pig liver Man₉-mannosidase, followed essentially the strategy recently elaborated for the isolation of the corresponding calf liver enzyme [3] with the following two alterations: (i) the pre-extraction of the crude microsomes with a low-salt/low-detergent buffer was omitted; (ii) a CM-Sepharose-binding step was added which preceded affinity chromatography as the initial purification step. This second change eliminated the need for large sample volumes in the affinity-chromatography step, and this modification proved to be important, improving substantially the effectiveness of the affinity step and the enzyme yield. Independent of the sequence of operations, however, affinity chromatography represented the key step in the purification procedure by which the highest purification factor was achieved.

The molecular mass of purified Man₉-mannosidase, as determined by SDS/PAGE under reducing conditions, was found to be 49 kDa. The proteolytic experiments carried out in the presence or absence of detergents, indicate, however, that the isolated 49 kDa enzyme is likely to be a stable and catalytically active fragment which has been generated by cleavage of a 65 kDa form of Man₉-mannosidase during purification. Furthermore, these studies strongly suggest that the 65 kDa form of pig liver Man₉-mannosidase is a transmembrane protein containing a cytosolically located polypeptide domain of

Fig. 4. Intact pig liver Man_6 -mannosidase is a transmembrane protein with a molecular mass of 65 kDa

Pig liver crude microsomes suspended in 0.1 M-phosphate buffer, pH 7.5, were incubated at 37 °C with trypsin at a proteinase/protein ratio of 1:100 (w/w) in the presence (a) or absence (b) of 0.3% Triton X-100. At given times, aliquots were taken from the incubation mixtures and proteolysis terminated by the addition of SDS/PAGE buffer and heating of the samples to 100 °C for 5 min. Cleavage products were then analysed by SDS/PAGE and immunoblotting, using the affinity-purified anti- $(\text{Man}_6\text{-mannosidase})$ antibody as a tool (for details, see the Materials and methods section). First lane, purified 49 kDa $\text{Man}_6\text{-mannosidase}$.

size at least 3 kDa. The isolation of the catalytically active 49 kDa enzyme fragment with an overall yield of more than 18% shows, on the other hand, that the intact and membrane-associated $\text{Man}_6\text{-mannosidase}$ contains a rather labile polypeptide region [apparently linking the (stable) catalytic domain with the membrane-spanning domain] which is accessible to, and easily cleaved by, trypsin or the proteinases abundant in the crude microsomal fraction. The loss of a hydrophobic, possibly the membrane-anchoring domain after proteolysis is indeed reflected by two observations: (i) Triton X-114 phase-partitioning experiments revealed an enrichment of the 65 kDa protein in the detergent phase, whereas the isolated 49 kDa enzyme partitioned mainly into the aqueous phase (results not shown); (ii) during the purification of $\text{Man}_6\text{-mannosidase}$, detergents were only required for the initial solubilization step. Marked susceptibility to non-specific proteolysis as observed here appears to be a general problem encountered with the isolation of intact forms of detergent-solubilized trimming glycosidases as indicated by studies on rat liver ER $\alpha 1,2$ -mannosidase [20], rat liver Golgi α -mannosidases [5,6,21], rabbit liver $\alpha 1,2$ -mannosidase [4] and trimming glucosidase II from rat liver and pig kidney [22,23]. Proteolytic degradation has, therefore, to be considered as a potential source of unreliability when differences in physical and enzymic properties are interpreted as discriminating criteria for independent trimming activities.

The enzymic properties exerted by the purified 49 kDa enzyme fragment closely resemble those of the corresponding calf liver enzyme, including the unique

substrate specificity, pH-dependence and inhibition by dMM and its *N*-alkyl derivatives (see Table 2). These observations, as well as preliminary immunological results (not shown), point to a close structural and functional relationship between both enzymes and suggest that they catalyse the same processing steps during *N*-linked oligosaccharide assembly. On the other hand, it is particularly the substrate specificity which discriminates both the pig and calf liver enzyme from other trimming $\alpha 1,2$ -mannosidases so far studied [2,4,6,24]. In this context it should be realized, however, that much of the available data relies on the use of only partially purified enzyme preparations incubated *in vitro* with free high-mannose oligosaccharides as substrates. It is still an open question whether, and to what extent, the substrate specificity *in vitro* differs from that of the enzyme embedded in its natural membrane environment and, probably more importantly, to what extent this substrate specificity may be modulated by the protein moiety to which the oligosaccharide chain(s) are *N*-glycosidically attached. At least some evidence for the importance of the polypeptide backbone in the control of oligosaccharide processing, comes from studies *in vitro* and *in vivo* on the action of ER $\alpha 1,2$ -mannosidase [2,19]. These studies showed that the soluble and purified form of ER mannosidase (apparently being a proteolytic cleavage product) hydrolyses, with little preference, mannose residues from high-mannose structures ranging in size from $\text{Man}_6\text{-GlcNAc}$ to $\text{Man}_5\text{-GlcNAc}$, whereas, in the intact cell system, the membrane-associated enzyme seems to catalyse the removal of only a single mannose

residue from the Man₉-GlcNAc₂ oligosaccharide of certain *N*-glycoproteins. There is also ample evidence, however, that data from studies *in vitro* as obtained here for pig liver Man₉-mannosidase, may be transferable to the situation *in vivo*. Thus Bischoff *et al.* [2] found that the high-mannose glycan chains of the ER-resident glycoprotein hydroxymethylglutaryl-CoA reductase are processed as far as to the Man₆-GlcNAc₂ stage. In addition, they observed that the corresponding processing activities were sensitive, in a similar fashion to Man₉-mannosidase, to inhibition by dMM. Hickman *et al.* [25] reported that newly synthesized *N*-glycoproteins (immunoglobulin A α -chains) with oligosaccharide structures containing eight, seven and six, but not five, mannose residues accumulated in MOCP 315 murine plasmacytoma cells when protein transport from the lumen of the ER into the Golgi compartment was blocked by carbonyl cyanide *m*-chlorophenylhydrazine. Similar results were obtained from pulse-chase studies of the biosynthesis of vesicular-stomatitis virus in HeLa cells [26]. These combined data strongly support the view that, in addition to the ER mannosidase identified and characterized by Kornfeld *et al.* [2,19,20], another α 1,2-mannosidase must exist in the lumen of the ER having exactly the substrate specificity which we have determined for pig (and calf) liver Man₉-mannosidase. Recent studies on the subcellular location of pig liver Man₉-mannosidase indicate that this enzyme may indeed be an ER-resident protein (E. Bause, J. Schweden & F. Roth, unpublished work). The obvious occurrence of at least three distinct α 1,2-mannosidases within the cell (ER mannosidase, ER Man₉-mannosidase, Golgi mannosidase IA/B), all acting on Man₉-oligosaccharides, but differing in their final products, suggests that mannose processing may follow different routes beyond the Man₉ stage. A possible biological function of this 'multiple-route strategy' might be to allow the cell the production of a spectrum of glycan structures necessary for further processing or transport of particular glycoproteins within, or between, cell compartments.

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