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Purification and comparison of the structures of human liver acidic α-D-mannosidases A and B

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Human liver α-D-mannosidases A and B were purified 11 500-fold and 2000-fold respectively. Both showed microheterogeneity when analysed by isoelectric focusing. α-D-Mannosidases A and B are immunologically identical but differ in their range of pI values, molecular masses, uptake into fibroblasts and subunit compositions. α-D-Mannosidase A consists of equimolar proportions of subunits of molecular masses 62 kDa and 26 kDa, which are linked by disulphide bridges in the intact enzyme. α-D-Mannosidase B also contains a small subunit, of molecular mass 26 kDa, and a variable mixture of larger subunits, of molecular masses 58 kDa and 62 kDa. The 62 kDa and 58 kDa subunits, but not the 26 kDa one, contain concanavalin A-recognizing glycans. The 58 kDa subunit has a lower pI, contains less high-mannose glycans but probably contains more mannose 6-phosphate than the 62 kDa subunit. It is postulated that the differences in structure and properties of α-D-mannosidases A and B are due to differences in the state of processing of the large subunit. This suggestion is consistent with a single locus on chromosome 19 for lysosomal α-D-mannosidase.

INTRODUCTION

Lysosomal enzymes are synthesized in the form of precursors that undergo post-translational modification during transport from their site of synthesis on ribosomes associated with the rough endoplasmic reticulum to the lysosomes (Hasilik & Neufeld, 1980). The conversion of a precursor into the mature intralysosomal enzyme involves proteolysis, glycosylation and phosphorylation and generates transient intermediate forms of the enzyme. The α-D-mannosidase (EC 3.2.1.24) extractable from human liver, fibroblasts and other tissues exists in two major forms, A and B, easily separable by ion-exchange chromatography (Carroll *et al.*, 1972). Both forms are absent in the lysosomal storage disease mannosidosis. The molecular basis of the multiple forms of human α-D-mannosidase is not known. It has been shown that in normal human fibroblasts the enzyme is synthesized in the form of a precursor of molecular mass 110 kDa, which is initially processed to polypeptides of molecular masses 63–67 kDa and 40–46 kDa (Pohlmann *et al.*, 1983). In the present investigation the subunit structures of human liver α-D-mannosidase A and B were investigated in an attempt to relate the multiple forms of the mature enzyme to the processing observed in the biosynthesis of the enzyme and to understand the enzymic defect in mannosidosis. As our previous methods of purification either appeared to favour the conversion of α-D-mannosidase A into α-D-mannosidase B (Phillips *et al.*, 1976) or gave relatively poor purification (Phillips *et al.*, 1975), a modified procedure that gives good yields of α-D-mannosidases A and B was used. A preliminary report of some of this work has been published (Cheng *et al.*, 1982).

MATERIALS AND METHODS

Enzyme assays

Acidic and neutral α-D-mannosidase activities were assayed with the fluorigenic substrate 4-methylumbelliferyl α-D-mannopyranoside (Koch–Light Laboratories, Haverhill, Suffolk, U.K.) as described previously (Phillips *et al.*, 1974). Activities measured at pH 4.0 and pH 6.5 were termed acidic and neutral α-D-mannosidase respectively. One unit of activity is that amount of enzyme that transforms 1 μmol of substrate/min under these conditions. Other potentially contaminating glycosidases were assayed with the appropriate 4-methylumbelliferyl glycoside as substrate (Phillips *et al.*, 1976).

Protein was assayed by the Folin method (Lowry *et al.*, 1951), with bovine serum albumin as a standard.

Purification of acidic α-D-mannosidases A and B (summarized in Table 1)

Step 1. Initial extraction and separation of α-D-mannosidases A and B. α-D-Mannosidase was purified from normal human liver up to the stage of affinity chromatography on concanavalin A–Sepharose essentially as described previously by us (Phillips *et al.*, 1976) and other authors (Grabowski *et al.*, 1980). α-D-Mannosidases A and B were then separated by ion-exchange chromatography on a column (17.5 cm × 2.2 cm diam.) of DEAE-cellulose (Whatman DE 32), equilibrated with 10 mM-sodium phosphate buffer, pH 6.0. The unbound activity, α-D-mannosidase A, and the activity eluted at 0.06 M-NaCl by an NaCl gradient, α-D-mannosidase B,

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were subsequently purified further separately by ion-exchange chromatography on CM-cellulose (Whatman CM 32) and DEAE-cellulose respectively (Phillips *et al.*, 1974). Samples were concentrated at various stages in the purification by vacuum-dialysis against the appropriate buffer.

Step 2. Removal of contaminating β-*N*-acetylhexosaminidase by affinity chromatography. Contaminating β-*N*-acetylhexosaminidase was removed by affinity chromatography in 10 mM-sodium phosphate buffer, pH 6.8, on 2-acetamido-*N*-(6-aminohexanoyl)-2-deoxy-β-glucopyranosylamine coupled to CNBr-activated Sepharose 4B (5 ml column) prepared by the method of Koshy *et al.* (1975) and kindly provided by Dr. J. Stirling. Partially purified α-D-mannosidases A and B were not retarded, whereas the *N*-acetylhexosaminidase was completely retained on the column.

Step 3. Preparative gel electrophoresis in a gradient of polyacrylamide. The final step in the purification was removal of contaminating proteins of a similar charge but different sizes by preparative electrophoresis in a gradient of polyacrylamide. Electrophoresis was carried out in slab gels (80 mm × 80 mm × 3 mm) containing a gradient of polyacrylamide (3–26%) (Gradipore; Universal Scientific, Woodford Green, Essex, U.K.). The gels were pre-electrophoresed for 15 min at 125 V, followed by 20 min at 70 V in a Uniscil gradient-pore electrophoresis apparatus. The buffer, which was pre-cooled to 4 °C, consisted 10.75 g of Tris, 5.04 g of boric acid and 0.93 g of EDTA per litre of distilled water. The samples of α-D-mannosidases A and B were concentrated to 1.2 ml on a Minicon B-15 concentrator (Amicon), made 20% (w/v) with respect to sucrose and applied to four gradient polyacrylamide gels each. Electrophoresis was carried out at 90 V until the samples had left the sample wells and entered the gradient gel, and was then continued at 125 V for 24 h. After this time the gels were removed, washed for 30 min at 4 °C in 0.1 M-citric acid, adjusted to pH 4.0 with 0.2 M-Na_2HPO_4 and then stained briefly for α-D-mannosidase activity by incubation at 37 °C in a mixture containing 1-naphthyl α-D-mannopyranoside (1 mg/ml) and diazo-4-chloro-*o*-anisidine (2 mg/ml) in 0.1 M-citrate/phosphate buffer, pH 4.0 (Phillips *et al.*, 1975). After localization of the active band, the enzyme was extracted from the gels by maceration of the acrylamide by passage through a syringe followed by homogenization in 3 ml (per gel) of 10 mM-sodium phosphate buffer, pH 6.0, in a 7 ml Dounce homogenizer (Wheaton Scientific). The homogenate was transferred to a silicone-treated Corex centrifuge tube and shaken with the buffer for 5 h at 4 °C to extract the protein. After centrifugation at 3300 *g* for 15 min the pellet was re-extracted in a similar manner after further periods of 12 h, 24 h and 36 h. The supernatants were combined and concentrated to yield the final preparations of α-D-mannosidases A and B.

Analytical electrophoretic techniques

Samples of purified α-D-mannosidases A and B were analysed by electrophoresis in a gradient of polyacrylamide as described above. One half of the gel was stained for activity and the other half was stained for protein by immersion in 0.02% Coomassie Brilliant Blue in 10% (v/v) acetic acid. It was destained in 10% (v/v) acetic acid at 60 °C until a clear background was obtained. The molecular masses of the purified enzymes were estimated by comparison with the mobilities of standard proteins thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

Polyacrylamide-gel electrophoresis in the presence of SDS was performed in 10% polyacrylamide gels (165 mm × 195 mm × 1.5 mm) by the procedure of Laemmli (1970). To ensure that disulphide bands were not re-formed, the reduced polypeptides were carboxymethylated with 0.5 M-iodoacetamide (Roberts & Lord, 1981). Reduction and carboxymethylation were specifically omitted in certain experiments. Protein bands were detected by staining with 0.05% Coomassie Brilliant Blue in acetic acid/propan-2-ol/water (1:1:3, by vol.). The gels were destained by repeated washing with acetic acid/ propan-2-ol/water (1:1:8, by vol.). Glycoproteins were detected by the method of Parish *et al.* (1977) with the use of concanavalin A (Pharmacia) and horseradish peroxidase (Sigma Chemical Co.). Standard proteins were myosin (200 kDa), phosphorylase *b* (94 kDa), pyruvate kinase (60 kDa), 3-phosphoglycerokinase (45 kDa), 3-phosphoglyceraldehyde dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and myoglobin (17 kDa).

Isoelectric focusing

Analytical isoelectric focusing over the pH range 3.5–10 (Pharmalytes; Pharmacia) was carried out in polyacrylamide gels (5%, w/v) in cylindrical glass tubes (120 mm × 2.5 mm diam.) essentially in accordance with the manufacturer's laboratory manual (Pharmacia). The pH gradient was measured with a pI calibration kit (Pharmacia) of proteins of known pI values. Protein and α-D-mannosidase activity were detected as described for electrophoretic procedures. Samples containing 15–20 μg of purified α-D-mannosidase A or α-D-mannosidase B were analysed. Two-dimensional separations were carried out by the procedure of O'Farrell (1975). Detection of protein by the silver staining was by the method of Morrissey (1981).

Gel filtration on Sephadex G-200

The molecular masses of purified α-D-mannosidases A and B were estimated by using a Sepahadex G-200 (32 cm × 2.6 cm diam.) column equilibrated in 10 mM-sodium phosphate buffer, pH 6.0, containing 0.1 M-NaCl and calibrated with the following standards: cytochrome *c* (12.4 kDa), horseradish peroxidase (49 kDa), bovine serum albumin (67 kDa), fructose bisphosphate aldolase (58 kDa), bovine α-globulin (effectively 205 kDa) and Blue Dextran 2000 (< 1000 kDa).

Preparation of antiserum and immunodiffusion

Antibodies to purified human liver α-D-mannosidase B were produced in rabbits by intradermal injections at multiple sites (0.2 mg/animal), and double immunodiffusion (Ouchterlony) was carried out as described previously (Phillips *et al.*, 1975).

Immunoprecipitation and analysis of polypeptides labelled *in vitro*

Proteins (60 μg of crude α-D-mannosidase or 5 μg of a marker protein) were labelled *in vitro* with tritium by

Table 1. Purification of human liver acidic α-D-mannosidases A and B

Both forms were co-purified up to the concanavalin A–Sepharose chromatography step. After separation on DEAE-cellulose the two forms were processed separately. In parentheses are given the purification factor and yield relative to amounts of α-D-mannosidase A or B in original homogenate, assuming a ratio of 1:3 for forms A and B. Abbreviation: ANAG–Sepharose, 2-acetamido-*N*-(6-aminohexanoyl)-2-deoxy-β-glucopyranosylamine coupled to CNBr-activated Sepharose 4B.

Purification step	Activity (units)	Protein (mg)	Sp. Activity (units/mg)	Yield (%)	Purification (fold)
Homogenate (1.9 kg of liver)	224	140025	0.0016	100	1
Supernatant	199	104737	0.0019	100	1.2
40–55%-satn. $(NH_4)_2SO_4$ precipitate	170	5667	0.030	76	19
Heat treatment (60 °C, 60 min)	152	2492	0.061	68	38
Concanavalin A–Sepahrose column	81	307	0.26	36	162.5
DEAE-cellulose column					
(1) (a) α-D-Mannosidase A	12	49	0.25	5.4	156
(b) CM-cellulose column	7.8	8.4	0.93	3.5	581
(c) ANAG–Sepharose column	7.4	4.5	0.64	3.3	1025
(d) Preparative gel	1.3	0.28	4.6	0.58 (2.3)	2875 (11500)
(2) (a) α-D-Mannosidase B	33	79	0.42	15	262.5
(b) DEAE-cellulose column	19.5	40	0.49	8.7	306
(c) ANAG–Sepharose column	18	29	0.62	8	387.5
(d) Preparative gel	3.6	1.45	2.5	1.6 (2.1)	1562.5 (2080)

reductive methylation with the use of formaldehyde and KB^3H_4 (> 3 Ci/mmol; Amersham International) as described by Kumarasamy & Symons (1979). Labelled α-D-mannosidase was immunoprecipitated as described by Kessler (1975) with either protein A-bearing *Staphylococcus aureus* (Sigma Chemical Co.) or protein A–Sepharose (Pharmacia). Labelled polypeptides were analysed by electrophoresis in the presence of SDS after reduction and alkylation. Fluorography (Bonner & Laskey, 1974) with the use of EnHance (New England Nuclear) and exposure at −70 °C to pre-flashed Fuji X-ray film for 1–6 weeks was used to detect the radioactive bands.

Uptake of α-D-mannosidase into enzyme-deficient fibroblasts

Fibroblasts from a patient with mannosidosis, a disease resulting from a deficiency of lysosomal α-D-mannosidase, were grown in culture as described previously (Burditt *et al.*, 1978). Sterilized solutions of partially purified α-D-mannosidases A and B in culture medium were added to the culture flasks. Cultures of the cells were harvested at intervals over 48 h, then washed thoroughly, and the α-D-mannosidase activity in extracts of the cells was measured. α-D-Mannosidase B was preincubated with alkaline phosphatase–agarose in some experiments.

RESULTS

Purification of α-D-mannosidases A and B (Table 1)

α-D-Mannosidases A and B were purified to specific activities of 4.6 units/mg and 2.5 units/mg respectively, with recoveries of 0.58% and 1.6%. However, as α-D-mannosidases A and B are consistently present in crude liver extracts in a ratio of approx. 1:3, this really represents purifications of 11500-fold and 2000-fold and yields of 2.3% and 2.1% respectively for α-D-mannosidases A and B. As can be seen from Table 1, the α-D-mannosidase A/B ratio remains constant throughout the purification.

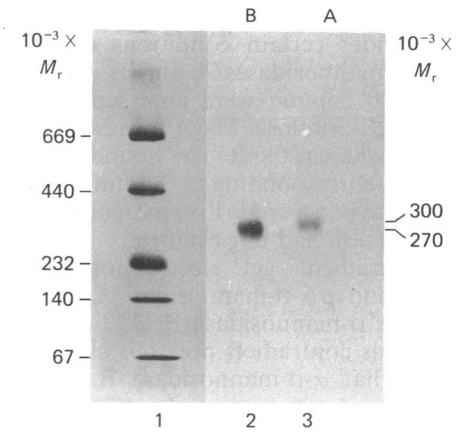

Fig. 1. Electrophoresis of purified α-D-mannosidases A and B in a gradient of polyacrylamide

Samples of purified α-D-mannosidase A (5 μg) and α-D-mannosidase B (10 μg) were electrophoresed for 48 h. The gel was stained for α-D-mannosidase, and subsequently lane 1 was stained for protein. Lane 1, standard proteins; lanes 2 and 3, α-D-mannosidases B and A respectively.

No neutral α-D-mannosidase activity was detected after the initial fractionation. As the acidic α-D-mannosidase is heat-stable, it was obtained free of most glycosidases by heating at 60 °C for 1 h. The only glycosidase contaminant before the penultimate step of the purification, β-*N*-acetylhexosaminidase, was removed by affinity chromatography. Analysis of the preparations after this stage by polyacrylamide-gel electrophoresis showed that α-D-mannosidase A contained three other proteins without α-D-mannosidase activity and that the α-D-mannosidase B preparation contained up to seven contaminants. These components were removed by

preparative electrophoresis in a gradient of polyacrylamide. The final preparations of α-D-mannosidases A and B consisted of single, albeit broad, enzymically active bands by analytical electrophoresis for 48 h in a gradient of polyacrylamide (Fig. 1). Duplicate gels showed only one protein band in each sample. The bands of activity were cut out and analysed by electrophoresis in 7% polyacrylamide, which again showed single protein bands by silver staining.

The degree of purification achieved here is higher than or comparable with values for human α-D-mannosidase from liver (Phillips *et al.*, 1976; Grabowski *et al.*, 1980), kidney (Marinkovic & Marinkovic, 1976) or placentae (Lee *et al.*, 1982; Khan & Basu, 1982). Rat liver (Opheim & Touster, 1978) and pig kidney (Okumura & Yamashina, 1973; Mersmann & Buddecke, 1977) acidic α-D-mannosidases have also been purified by similar procedures.

Characterization of purified α-D-mannosidases A and B

Estimation of molecular mass by gel filtration. Gel filtration of samples of purified α-D-mannosidases A and B on Sephadex G-200 suggested that α-D-mannosidase A had a higher molecular mass (280 ± 10 kDa) than α-D-mannosidase B (260 ± 10 kDa). As it has been suggested previously that α-D-mannosidases A and B interconvert under certain conditions (Phillips *et al.*, 1974), the α-D-mannosidases A and B eluted from the Sephadex G-200 column were analysed by chromatography on DEAE-cellulose. The A form was not retarded by the column, whereas the B form bound and was eluted at 0.06 M-NaCl, corresponding to the elution position for α-D-mannosidase B in a crude liver extract. This indicated that interconversion had not occurred.

Analytical gradient gel electrophoresis had also suggested that native α-D-mannosidase A (300 ± 20 kDa) is larger than α-D-mannosidase B (270 ± 25 kDa) (Fig. 1). However, this contradicts previous claims, based on gel filtration, that α-D-mannosidase B is larger than α-D-mannosidase A (Phillips *et al.*, 1974; Grabowski *et al.*, 1980). The probable explanation of this discrepancy is the omission of NaCl from the buffer used for gel filtration in the earlier reports. Sephadex is known to contain a small number of carboxy groups, which bind charged solutes at low ionic strength. Thus at pH 6.0 the negatively charged α-D-mannosidase B would not be retarded whereas α-D-mannosidase A (pI 6.2–6.75) could be retarded.

Molecular masses of 205 kDa and 300 kDa have been estimated for placental α-D-mannosidase, which is a mixture of forms A and B, by sedimentation equilibrium (Lee *et al.*, 1982) and gel filtration (Khan & Basu, 1982) respectively.

Subunit composition. The subunits of pure α-D-mannosidases A and B were detected by polyacrylamide-gel electrophoresis in the presence of SDS after reductive carboxymethylation (Fig. 2). Under these conditions α-D-mannosidase A was resolved into two bands of apparent molecular masses 62 kDa and 26 kDa and the α-D-mannosidase B into three bands with apparent molecular masses of 62 kDa, 58 kDa and 26 kDa. The sizes of the subunits for the two forms are consistent with the observation that native α-D-mannosidase is larger than α-D-mannosidase B. The patterns of the bands were

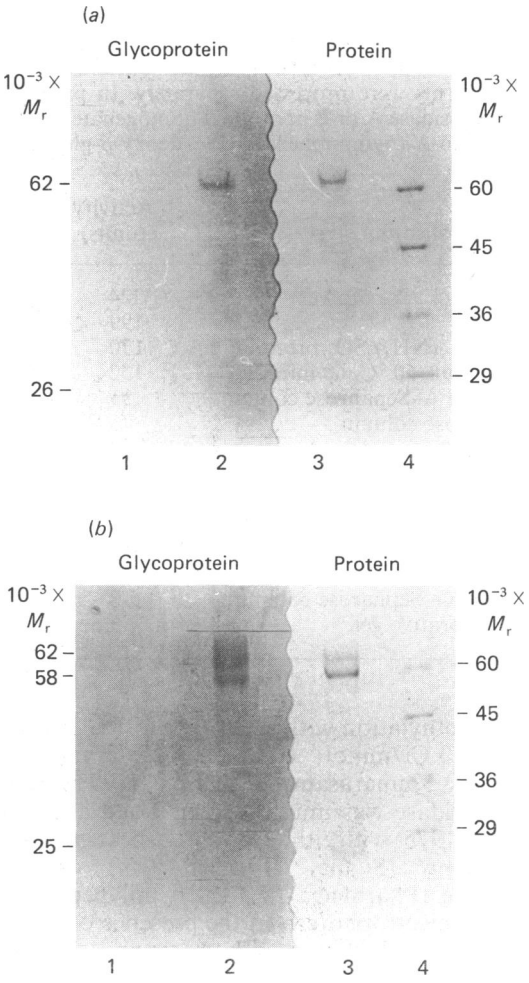

Fig. 2. Polyacrylamide-gel electrophoresis of purified α-D-mannosidases A and B in the presence of SDS

Duplicate samples (20 μg) of pure α-D-mannosidase A and α-D-mannosidase B were analysed by electrophoresis in polyacrylamide (10%, w/v) containing SDS. The gels were divided into two, and one half was stained for protein with Coomassie Blue and the other for glycoprotein with the use of concanavalin A/peroxidase. (*a*) Lanes 2 and 3, α-D-mannosidase A; lanes 1 and 4, marker proteins. (*b*) Lanes 2 and 3, α-D-mannosidase B; lanes 1 and 4, marker proteins.

unaffected either by increasing the concentration of the reducing agent up to a concentration of 50 mM-dithiothreitol or by increasing the time of heating. This suggested that reduction of the enzyme was complete under the conditions used.

The stoichiometry of the subunits in α-D-mannosidases A and B was determined by scanning the Coomassie Blue-stained gels with the densitometer attachment on a Pye–Unicam SP. 1800 recording spectrophotometer. This procedure was validated by analysing the subunits observed when denatured and reduced human γ-globulin was electrophoresed under the same conditions. The ratio of the areas of the peaks corresponding to the heavy (55 kDa) and light (25 kDa) chains was 2:1. The theoretical ratio if the amount of bound Coomassie Blue was proportional to the size of the polypeptide would be 2.2:1. Therefore this procedure does give a reasonable

estimate of the relative proportions of polypeptides of these sizes.

The densitometric scan of the Coomassie Blue-stained subunits of α-D-mannosidase A contained two peaks corresponding to the two subunits of molecular masses 62 kDa and 26 kDa. The areas under the peaks were in the ratio 2.3:1. As the estimated molecular mass of the larger subunits was 2.4-fold greater than that of the smaller subunit, this suggests that the subunits are present in equimolar proportions.

Three peaks were observed in the scan of the subunits of α-D-mannosidase B, corresponding to polypeptides with molecular masses of 62 kDa, 58 kDa and 26 kDa. The ratio of the area under the combined peaks of molecular masses 58 and 62 kDa to the area under the peak of molecular mass 26 kDa was 2.4:1. Although the proportions of the 58 kDa and 62 kDa subunits varied from one preparation to another, this again indicates that the small and the large subunits are present in equal proportions.

To investigate whether the subunits were linked by disulphide bridges in the native enzyme, α-D-mannosidase A and B preparations that had been unfolded but not reduced and carboxymethylated were analysed by electrophoresis in a gradient of polyacrylamide gel in the presence of SDS. Two components were observed in both α-D-mannosidase A and α-D-mannosidase B. One had an estimated molecular mass of 255 kDa and the other a molecular mass of 58 kDa. This shows that the small subunits but not all the large subunits are involved in disulphide bridges in the native enzyme or not released under the conditions used. Extraction of the 255 kDa and 58 kDa components from the denatured enzymes, A and B, and subsequent reduction and analysis by electrophoresis yielded polypeptides of 24–26, 58 and 62 kDa and of 58 and 62 kDa respectively. Subunits of molecular masses 58 kDa and 30 kDa have been reported to be present in human kidney α-D-mannosidase A (Marinkovic & Marinkovic, 1976).

Detection of carbohydrate on the subunits of human liver α-D-mannosidases A and B. The subunits of human liver α-D-mannosidases A and B separated by electrophoresis on a polyacrylamide gel were stained for glycoprotein by the concanavalin A/peroxidase method (Parish *et al.*, 1977). In α-D-mannosidase A the component with molecular mass 62 kDa was heavily stained for glycoprotein but the 26 kDa subunit was unstained, even with a high loading of protein (Fig. 2*a*). In α-D-mannosidase B both the 62 kDa and 58 kDa subunits stained positively for glycoprotein but not the 26 kDa subunit (Fig. 2*b*). Although the protein stain showed that the amount of 58 kDa subunit was greater than the amount of 62 kDa subunit in α-D-mannosidase B, the subunits stained with approximately equal intensity for carbohydrate. This suggests that the 62 kDa component in α-D-mannosidase B contains more concanavalin A-recognizing glycans than does the 58 kDa component.

Isoelectric focusing. Analytical isoelectric focusing in polyacrylamide of the purified α-D-mannosidases A and B revealed microheterogeneity in both forms. The patterns of bands were identical when stained for protein or α-D-mannosidase activity, indicating the absence of any major non-enzymic contaminants. Eight enzymically active bands were detected in α-D-mannosidase A. The main components had pI values of 6.2 and 6.75 with minor components in the range pI 6.1–6.85. α-D-Mannosidase B contained nine detectable enzymically active bands with the main components having pI values of 5.4 and 5.45 and minor components with pI values between 5.05 and 5.6. This microheterogeneity, which has been observed previously in purified human α-D-mannosidase (Phillips *et al.*, 1974; Lee *et al.*, 1982), probably accounts for the diffuse activity bands observed for the native enzyme after electrophoresis.

To see whether there was any relationship between this observed charge heterogeneity and the subunit composition, α-D-mannosidases A and B were analysed by two-dimensional electrophoresis. After isoelectric focusing under reducing and denaturing conditions the subunits were analysed by electrophoresis in the presence of SDS in the second dimension. The large subunit in α-D-mannosidase B occurred in multiple forms with different pI values. There was a clear correlation between the pI of the focused polypeptides and their size: the lower the pI, the greater the proportion of the 58 kDa subunit. Similarly the large subunit in α-D-mannosidase A showed charge heterogeneity but all of the polypeptides had a molecular mass of 62 kDa. Thus the pI values of the native forms A and B appear to be determined by the pI of the larger subunit.

No small subunits were detected in these experiments, either because they focused out of the pH range or more probably because they were not detected by the silver staining procedure.

Uptake of α-D-mannosidases A and B into α-D-mannosidase-deficient fibroblasts. α-D-Mannosidase activity was taken up into α-D-mannosidosis fibroblasts when α-D-mannosidase B was added to the culture medium, but not when α-D-mannosidase A was added to the medium (Fig. 3). The uptake of α-D-mannosidase B at 24 h was inhibited by 84% when 0.1 mM-mannose 6-phosphate was also included in the medium and by 67% when the liver α-D-mannosidase B was preincubated with alkaline phosphatase. This suggests that α-D-mannosidase B, or at least some components of this form, contain mannose 6-phosphate, the lysosomal recognition marker.

Immunological characterization of α-D-mannosidases A and B. Purified α-D-mannosidases A and B were shown to be immunologically identical by immunodiffusion and immunoprecipitation with the antiserum raised against the pure α-D-mannosidase B. Immunological identity (Phillips *et al.* 1975) and cross-reactivity (Grabowski *et al.*, 1980) between human liver α-D-mannosidases A and B have been observed previously. This suggests the two forms are structurally and genetically related, which is consistent with the single locus on chromosome 19 for human α-D-mannosidase (Champion & Shows, 1977) and their concomitant deficiency in α-mannosidosis (Carroll *et al.*, 1972).

To investigate whether the subunits detected in the purified forms of the enzyme were also present in impure samples of the enzyme and not artifacts of the purification procedure, labelled polypeptides were immunoprecipitated from partially purified enzyme and crude tissue extracts. A sample of human liver acidic α-D-mannosidase that had been purified approx. 100-fold up to the stage of affinity chromatography on concanavalin A–Sepharose in the purification procedure was labelled

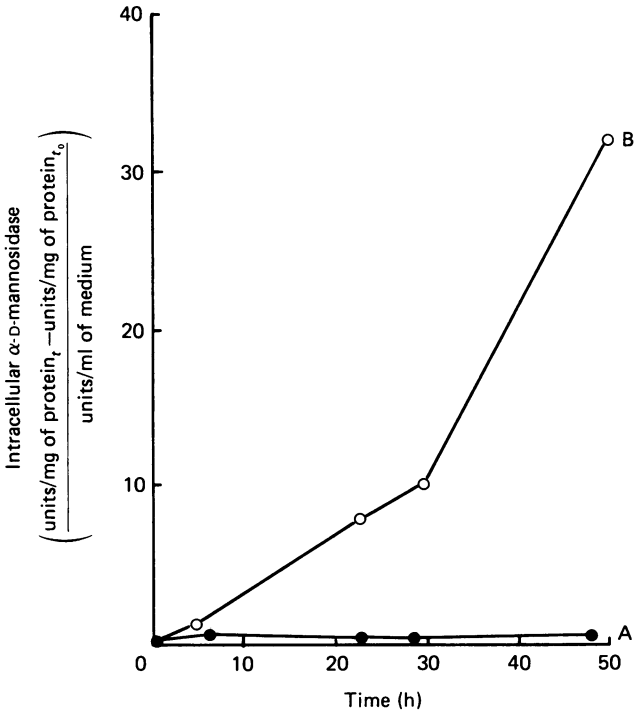

Fig. 3. Uptake of α-D-mannosidases A and B into enzyme-deficient fibroblasts

Partially purified human liver α-D-mannosidase A (●) and α-D-mannosidase B (○) were included in separate cultures of human fibroblasts deficient in α-D-mannosidase. At different times the cells were harvested and the α-D-mannosidase activity in the cells was measured. The increase in intracellular α-D-mannosidase activity was calculated by subtracting the intracellular specific α-D-mannosidase activity at $t = 0$ from the specific activity at a particular time, t, and dividing this value by the initial concentration of the exogenous liver α-D-mannosidase in the medium. Average values from a representative experiment are shown.

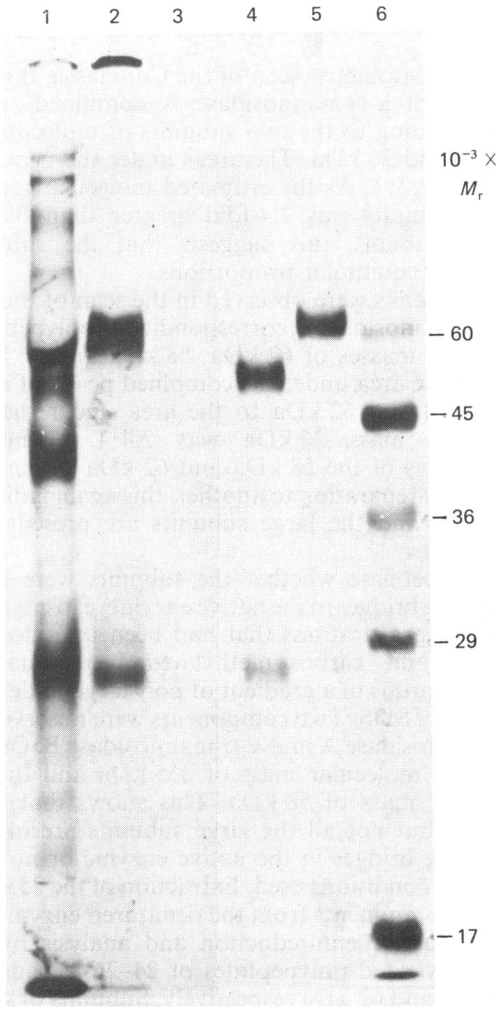

Fig. 4. Immunoprecipitation of α-D-mannosidase that had been labelled *in vitro*

Partially purified α-D-mannosidase that had been labelled *in vitro* with KB^3H_4 was immunoprecipitated with antiserum and protein A–Sepharose. The labelled polypeptides were analysed by electrophoresis in polyacrylamide (10%, w/v) and located by fluorography. Lanes 1 and 2, α-D-mannosidase that had been labelled *in vitro* before and after immunoprecipitation; lane 3, immunoprecipitation with non-immune serum; lanes 4 and 5, tritiated human IgG and serum albumin respectively; lane 6, tritiated molecular-mass markers.

in vitro with ^3H by reductive methylation. It contained α-D-mannosidases A and B. Labelled polypeptides were precipitated from this sample by addition of antiserum and protein A–Sepharose. The labelled polypeptides were solubilized, reduced and denatured with SDS before analysis by electrophoresis followed by detection by fluorography (Fig. 4). Less than 1% of the labelled protein was precipitated. Only two radioactive bands, estimated to have molecular masses of 58–62 kDa and 25–26 kDa, were detected. The two observed bands were not precipitated by non-immune serum. They had the same mobilities as the subunits of purified but unlabelled α-D-mannosidases A and B that had been stained with Coomassie Blue (not shown). It is concluded that the larger and the smaller immunoprecipitated bands consist of a mixture of the subunits of molecular masses 62 kDa and 58 kDa and the subunit of molecular mass 26 kDa respectively. This would account for the thickness of the bands and be consistent with the presence of both α-D-mannosidases A and B and the specificity of the antiserum. The same bands were also immunoprecipitated from crude human liver extracts labelled *in vitro*. This observation confirms the presence of large and small subunits in impure α-D-mannosidases A and B and demonstrates that they are not artifacts of the purification procedure.

DISCUSSION

Our results show that native α-D-mannosidases A and B are immunologically identical but differ in their apparent molecular masses, isoelectric points, affinities for the lysosomal mannose 6-phosphate receptor and subunit compositions. The structural feature(s) that determine(s) whether α-D-mannosidase is the A or the B form must carry charge, as forms A and B are readily

separated by ion-exchange chromatography and their pI values do not overlap.

Both forms contain a small subunit of approx. 26 kDa that is linked by disulphide bridges in the native forms. Although this subunit consistently appeared to be slightly smaller in the B form than in the A form, the main difference between the two forms was in the nature of the larger subunits, of 62 kDa in form A and a mixture of 58 kDa and 62 kDa in form B. Both large subunits show charge microheterogeneity by isoelectric focusing, but the range of pI values for the 58 kDa subunit is lower than that for the 62 kDa subunit. Thus it is probably a structural feature on the large subunit that accounts for the difference in charge and other properties between forms A and B. However, the two types of large subunits are very closely related structurally, because the 62 kDa subunit can be generated by reduction from the 58 kDa subunit obtained from the denatured but non-reduced intact enzyme. Thus it is possible that the structural feature that distinguishes the two large subunits may be modified physiologically or chemically *in vitro*. This would explain the variable proportions of the two large subunits found in preparations of reduced α-D-mannosidase B, the reported interconversion of α-D-mannosidases A and B and the detection of the 58 kDa subunit in denatured α-D-mannosidase A (which contained only the 62 kDa subunit after denaturation and reduction). The 58 kDa subunit contains less high-mannose chains than the 62 kDa subunit, but contains more mannose 6-phosphate, because of the greater affinity of α-D-mannosidase B for the lysosomal receptor. Thus the charge difference could be due to the number or state of esterification of phosphate groups on the high-mannose chains. The recognition marker for the delivery of lysosomal enzyme precursors to lysosomes has been shown to be mannose 6-phosphate residues in high-mannose oligosaccharides linked via asparagine to the polypeptide chain (Kaplan *et al.*, 1977). The absence of high-mannose oligosaccharides, which recognize concanavalin A, on the small subunit in both α-D-mannosidases A and B suggests that these polypeptides are transported to the lysosomes in association with the larger subunits. This is consistent with the lack of detection of smaller subunits after electrophoresis of the denatured but non-reduced enzyme. Phosphorylated polypeptides of molecular mass 58–64 kDa were found in human fibroblasts by specific immunoprecipitation with anti-(human liver α-D-mannosidase B) serum (Pohlmann *et al.*, 1983). The mannose 6-phosphate could be present on the 58 kDa subunit. This would be consistent with the lower pI of this polypeptide, the presence of concanavalin A-binding glycans and the detection of phosphate on the processing intermediates of molecular mass 58–64 kDa in fibroblasts. The form of pig kidney α-D-mannosidase with the lowest pI is also the most rapidly endocytosed form of the enzyme (Mersmann *et al.*, 1978).

A difference in sialic acid content could be an alternative explanation of the charge difference between α-D-mannosidases A and B. Both human liver α-D-mannosidases A and B contain sialic acid and can be converted into a more basic form by treatment with neuraminidase (Chester *et al.*, 1975; Phillips *et al.*, 1976). However, treatment with neuraminidase did not destroy the receptor-mediated endocytosis of pig kidney α-D-mannosidase B_2 (Mersmann *et al.*, 1978). It is suggested that sialylation could be responsible for the microheterogeneity observed in α-D-mannosidases A and B but that the presence of some 58 kDa subunit containing mannose 6-phosphate determines whether the enzyme is A or B. Differences in the nature of asparagine-linked glycans in a single lysosomal enzyme are well documented (Hasilik & von Figura, 1981; Mathur *et al.*, 1984). Thus the molecular basis of the multiple forms of human lysosomal α-D-mannosidase is the different processing of the large subunit. This information on the subunit structure of α-D-mannosidase clarifies the enzymic defect in mannosidosis and will help in identifying cloned recombinant DNA sequences and coding for enzyme.

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REFERENCES

Bonner, W. L. & Laskey, R. (1974) Eur. J. Biochem. **46**, 83–88
Burditt, L. J., Chotai, K. A. & Winchester, B. G. (1978) FEBS Lett. **91**, 186–189
Carroll, M., Dance, N., Masson, P. K., Robinson, D. & Winchester, B. G. (1972) Biochem. Biophys. Res. Commun. **49**, 579–583
Champion, M. J. & Shows, T. B. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2968–2972
Cheng, S., Malcolm, S., Pemble, S. & Winchester, B. (1982) Biochem. Soc. Trans. **10**, 403–404
Chester, M. A., Lundblad, A. & Masson, P. K. (1975) Biochim. Biophys. Acta **391**, 341–348
Grabowski, G., Ikonne, J. U. & Desnick, R. J. (1980) Enzyme **25**, 13–25
Hasilik, A. & Neufeld, E. F. (1980) J. Biol. Chem. **255**, 4937–4945
Hasilik, A. & von Figura, K. (1981) Eur. J. Biochem. **121**, 125–129
Kaplan, A., Achord, D. T. & Sly, W. S. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2026–2030
Kessler, S. W. (1975) J. Immunol. **115**, 1617–1624
Khan, F. A. & Basu, D. (1982), J. Biosci. **4**, 133–138
Koshy, A., Robinson, D. & Stirling, J. L. (1975) Biochem. Soc. Trans. **3**, 244–246
Kumarasamy, R. & Symons, R. H. (1979) Anal. Biochem. **95**, 359–363
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lee, J. E. S., Little, T. E. & Yoshida, A. (1982) Enzyme **28**, 33–40
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Marinkovic, D. V. & Marinkovic, J. N. (1976) Biochem. J. **155**, 217–223
Mathur, R., Alvares, K. & Balasubramanian, A. S. (1984) Biochem. Biophys. Res. Commun. **123**, 1185–1193
Mersmann, G. & Buddecke, E. (1977) Int. J. Biochem. **8**, 763–768
Mersmann, G., Becker, R. & Buddecke, E. (1978) Biochim. Biophys. Acta **525**, 154–161
Morrissey, J. H. (1981) Anal. Biochem. **117**, 307–310
O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021
Okumura, T. & Yamashina, I. (1973) J. Biochem. (Tokyo) **73**, 131–138
Opheim, D. J. & Touster, O. (1978) J. Biol. Chem. **253**, 1017–1023
Parish, R. W., Schmidlin, S. & Muller, U. (1977) Exp. Cell Res. **110**, 267–276

Phillips, N. C., Robinson, D. & Winchester, B. G. (1974) Clin. Chim. Acta **55**, 11–19

Phillips, N., Robinson, D. & Winchester, B. (1975) Biochem. J. **151**, 469–475

Phillips, N. C., Robinson, D. & Winchester, B. G. (1976) Biochem. J. **153**, 579–587

Pohlmann, R., Hasilik, A., Cheng, S., Pemble, S., Winchester, B. & von Figura, K. (1983) Biochem. Biophys. Res. Commun. **115**, 1083–1089

Roberts, L. M. & Lord, J. M. (1981) Eur. J. Biochem. **119**, 31–41

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