RESEARCH COMMUNICATION

Partial sequence of the purified protein confirms the identity of cDNA coding for human lysosomal α -mannosidase B

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Human lysosomal α -mannosidase has been purified by a simple and rapid method in sufficient quantities for the analysis of its subunit composition and partial protein sequencing. Analysis of the N-terminal residues of the 30 kDa polypeptide has enabled us to confirm the identity of the recently cloned cDNA that was tentatively identified as that of lysosomal α -mannosidase [Nebes

and Schmidt (1994) *Biochem. Biophys. Res. Commun.* **200**, 239–245] and to locate the position of this polypeptide within the total deduced amino acid sequence. This finding will therefore provide a firm foundation for the characterization of α -mannosidosis mutations.

INTRODUCTION

Lysosomal α -D-mannosidase (EC 3.2.1.24) exists in two major forms, A and B, in human tissues and body fluids [1,2]. Both these forms arise from a common 110 kDa precursor [3] and the gene coding for these isoenzymes, *MAN B*, has been mapped to the long arm of chromosome 19 [4]. Mutations at this locus cause an autosomal recessive storage disease, α -mannosidosis, in several species, including man [5]. Studies of α -mannosidase at the molecular level, and the nature of the mutations in α -mannosidosis have lagged behind similar studies of other lysosomal enzymes. This is largely because purification of α -mannosidase has proved difficult, given the relatively low abundance of the enzyme in tissues from which purification has been attempted.

The amino acid sequence of the human-lysosomal- α -mannosidase precursor has recently been deduced by PCR-mediated cloning and sequencing of cDNA [6]. However, the identity of this cDNA depended on its similarity to that of the cDNA of the *Dictyostelium discoideum* (cellular slime mould) lysosomal enzyme and on its chromosomal localization, rather than on direct evidence such as expression or comparison of the deduced amino acid sequence with that of the purified enzyme. Direct evidence to establish the identity of the human lysosomal α -mannosidase cDNA is desirable because rat endoplasmic reticulum, yeast vacuolar, mouse Golgi, slime-mould and human lysosomal α -mannosidases all share a region of sequence similarity that might include active-site residues [1].

In the present work we have purified α -mannosidase B from the human leukaemic cell line HL 60 by using a simple and rapid purification procedure that exploits the remarkable thermostability of the enzyme. Four polypeptides were obtained on SDS/PAGE (67, 59, 55 and 30 kDa), the smallest of which yielded an N-terminal sequence matching exactly that predicted from the cDNA and located the 30 kDa fragment towards the C-terminal end of the precursor polypeptide sequence.

MATERIALS AND METHODS

RPMI 1640 medium and fetal-calf serum were obtained from Gibco Europe, Breda, The Netherlands. 4-Methylumbelliferyl α -

mannopyranoside (4MU α Man), 4-methylumbelliferone and Fast Red GG salt were from Sigma, St. Louis, MO, U.S.A. DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. The Bio-Rad protein assay, silver stain kit and SDS/PAGE molecular-mass standards were from Bio-Rad Laboratoires, Richmond, CA, U.S.A. The substrate 1-naphthyl α -D-mannopyranoside was from Melford Laboratories, Chelsworth Ipswich, Suffolk, U.K. All other reagents were of analytical grade.

HL 60 cell culture

The HL 60 cell line [7] was cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal-calf serum, in a humidified atmosphere containing 5% CO₂.

Enzyme assay and protein determination

Enzyme activity was measured by using 3 mM 4MU α Man in 0.2 M acetic acid/0.2 M sodium acetate buffer, pH 4.0 (buffer A). The fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin-Elmer LS3 fluorimeter (excitation at 360 nm; emission at 446 nm). One unit is the amount of enzyme that hydrolyses 1 μ mol of substrate/min at 37 °C. The protein content was determined by the Bradford method [8], using crystalline bovine γ -globulin as the standard. Specific activity was expressed as enzyme units/mg of protein.

Purification of lysosomal α -mannosidase B

All purification procedures were carried out at 4 °C unless otherwise stated.

Step 1: preparation of cell lysate and enzyme extraction

HL-60 cells (5×10^9) were harvested from culture medium by centrifugation (500 g for 10 min in an MSE bench-top centrifuge), washed three times with 100 ml of 0.9% NaCl and resuspended in 100 ml of 10 mM sodium phosphate buffer, pH 6.0, containing 0.1% (v/v) Miranol H2M detergent, then centrifuged at 17000 g for 20 min in a Sorvall RC-5B centrifuge.

Abbreviation used: 4MU α Man, 4-methylumbelliferyl α -mannopyranoside.

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Step 2: separation of the two α -mannosidase isoenzymes

The supernatant was loaded on to a DEAE-cellulose column (10 ml volume) equilibrated with 10 mM sodium phosphate buffer, pH 6.0, and separation performed as described in [9]. After washing the column until the A_{280} reached zero, the column was developed with a linear gradient of NaCl (0.0–0.3 M) in 120 ml of the above buffer and finally with 1.0 M NaCl in the same buffer. Fractions (2.5 ml each) were collected and assayed for their α -mannosidase content.

Step 3: heat treatment

Before heat treatment, the enzymically active peaks from DEAE-cellulose chromatography, corresponding to α -mannosidases A and B, in order of their elution, were pooled separately and incubated at 37 °C for 30 min in the presence of 1 mM ZnSO₄, followed by incubation at 70 °C for 60 min. After a final incubation at 4 °C for 90 min and centrifugation at 17000 *g* for 10 min, pellets were discarded and supernatants were dialysed against water and concentrated by vacuum dialysis in a Sartorius (Gottingen, Germany) apparatus.

PAGE

The purity and the molecular masses of the native proteins were determined by non-denaturing 5–20 %-polyacrylamide-gradient-gel electrophoresis (Bio-Rad Mini-Protean II apparatus). The gel was pre-electrophoresed for 15 min at 20 mA. The running buffer was 0.025 M Tris/0.192 M glycine, pH 8.3. Samples were concentrated and dialysed against water and were made 20 % (w/v) with respect to sucrose and applied to the gel. Electrophoresis was carried out at 20 mA for 5 h. For α -mannosidase activity staining, one half of the gel was soaked in buffer A, then incubated at 37 °C for 30 min in a mixture containing 1-naphthyl α -D-mannopyranoside (1 mg/ml) and Fast Red GG salt (2 mg/ml) in buffer A. Protein bands were detected by staining the other half of the gel with 0.1 % Coomassie Brilliant Blue in methanol/acetic acid/water (4:1:5, by vol.). SDS/PAGE was performed in 12 %-polyacrylamide gels (Bio-Rad Mini-Protean II apparatus) as described in [10]. Proteins were silver-stained according to the manufacturer's instructions.

Kinetic properties and thermal stabilities

pH optima were determined in 0.2 M acetic acid/0.2 M sodium acetate buffers in the pH range 3.0–6.5. K_m values were determined by Lineweaver–Burk plots [11] using the fluorogenic substrate 4MU α Man over the concentration range 0.27–2.27 mM.

Thermal stability of isoenzymes was determined, in the absence and in the presence of 1 mM ZnSO₄, by incubating samples (50 μ l) in buffer A at various temperatures ranging from 37 to 85 °C for 60 min. Samples were then cooled on ice for 2 h and assayed for enzyme activity at 37 °C in the usual way. The results were expressed as the percentage of enzyme activity found in non-heated controls kept on ice.

Sequence determination

The purified protein (10 mg) was fractionated by SDS/12 %-PAGE and electroblotted on to ProBlott membranes (Applied Biosystems) in 10 mM 3-(cyclohexyl)propane-1-sulphonic acid ('CAPS'), pH 11, containing 10 % methanol [12]. Membranes were stained with 0.1 % Coomassie Blue R250 in 50 % methanol and destained in 50 % methanol. The band corresponding to isoenzyme B (30 kDa) was cut out and sequenced in an Applied Biosystems model 476A sequencer, using a Blott Cartridge (Applied Biosystems) with an optimized liquid-phase program. Sequence determination was confirmed employing two independent protein preparations.

RESULTS AND DISCUSSION

HL 60 cells exhibited a relatively high α -mannosidase activity when compared with the human sources examined so far, and were found to be an abundant source of protein.

By exploiting an effective method for the separation of the α -mannosidase isoenzymes and their remarkable heat-stability in the presence of Zn²⁺, we developed a rapid and simple enzyme purification procedure (summarized in Table 1) which yielded pure α -mannosidase B and highly purified α -mannosidase A. α -Mannosidases A and B can be easily separated by DEAE-cellulose chromatography (Figure 1). The two forms displayed the same optimum pH of 4.0 and had similar pH-activity curves. K_m (mM) values, using 4MU α Man as the substrate, were

Table 1 Purification of lysosomal α -mannosidases A and B from HL 60 cells

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units)	Yield (%)	Purification (fold)
Cell lysate (5×10^9 cells)	458.00	5.50	0.012	100	1.00
Supernatant (17 000 <i>g</i>)	299.44	5.39	0.018	98	1.50
DEAE-cellulose chromatography*					
(1) α -Mannosidase A	16.79	2.43	0.145	44	12.07
(a) Zn ²⁺ treatment (37 °C, 30 min)	16.79	2.75	0.164	50	13.67
(b) Heat treatment (70 °C, 60 min)	0.47	2.47	5.255	45	437.94
(2) α -Mannosidase B	5.73	1.95	0.340	36	28.36
(a) Zn ²⁺ treatment (37 °C, 30 min)	5.73	2.09	0.365	38	30.42
(b) Heat treatment (70 °C, 60 min)	0.15	1.86	12.400	34	1033.33

* Both isoenzymes were co-purified up to the DEAE-cellulose chromatography step, then processed separately.

Figure 1 DEAE-cellulose chromatography of α -mannosidase from HL 60 cell extract

The cell extract was applied to a DEAE-cellulose column equilibrated with 10 mM sodium phosphate buffer, pH 6.0, and eluted with the same buffer until the peak of unretained protein was collected. Protein retained by the column was eluted with a linear gradient of 0–0.3 M NaCl and finally with 1.0 M NaCl. Fractions were assayed for activity towards the substrate 4MU α Man.

Figure 2 Thermal stability of α -mannosidases A and B determined in the absence (●) or presence (○) of 1 mM ZnSO₄

Samples were incubated for 60 min at the indicated temperatures, then cooled on ice for 2 h and assayed for enzyme activity. Results are means of three independent experiments and are expressed as a percentage of the activity at 37 °C.

0.91 ± 0.25 for α -mannosidase A and 1.33 ± 0.14 for α -mannosidase B. Both the isoenzymes were stable when heated at temperatures up to 60 °C. This range of thermal stability was increased up to 70 °C when the isoenzymes were preincubated for 30 min at 37 °C with 1 mM ZnSO₄ (Figure 2). Initial separation of the isoenzymes by DEAE-cellulose chromatography proved to be an indispensable step, and the reproducibility of the method required the sequential treatment of the separated isoenzymes with Zn²⁺, heat and centrifugation to remove denatured protein. No 'neutral' α -mannosidase was detected in the final preparations. K_m values, thermal stability and the behaviour of α -mannosidases A and B on DEAE-cellulose were identical with those of the partially purified isoenzymes. We found no evidence of conversion of the A into the B form that was reported to take place under certain conditions [13].

Figure 3 Electrophoresis of purified α -mannosidase A and B in a 5–20% gradient polyacrylamide gel

Samples (5 μ g) of purified isoenzymes were electrophoresed under non-denaturing conditions as described in the Materials and methods section. Lanes 1 and 2, α -mannosidases B and A stained for enzyme activity; lanes 3 and 4, isoenzyme B and standard proteins stained with Coomassie Blue. Molecular-mass (M) standard proteins were BSA monomer (66 kDa) and dimer (132 kDa), β -amylase (200 kDa) and jack-bean (*Canavalia ensiformis*) urease (272 kDa).

Figure 4 SDS/PAGE of purified α -mannosidase B

Pure isoenzyme (5 μ g) was analysed by electrophoresis in 12%-polyacrylamide gel in the presence of SDS. Protein bands were detected by silver staining. Lane 1, α -mannosidase B; lane 2, molecular-mass (M) marker proteins: phosphorylase b (97.4 kDa), BSA (66.2 kDa), bovine carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa) and hen's-egg-white lysozyme (14.4 kDa).

The final preparation of α -mannosidase B consisted of a single enzymically active protein band (Figure 3, lanes 1 and 3) when subjected to analytical gel electrophoresis in a 5–20%-polyacrylamide gradient. A single protein band was also detected on electrophoresis on a 6.5%-polyacrylamide gel (results not shown). The final preparation of α -mannosidase A had a major band of protein coincident with the staining for enzyme activity, but, in addition, there were a few minor contaminants. Specific-activity values for both α -mannosidases, A and B, were higher than those reported for α -mannosidases from human liver [2], kidney [14] and placenta [15].

Estimates of molecular mass from gradient-gel electrophoresis gave values of 296 kDa for α -mannosidase A and 250 kDa for α -mannosidase B (Figure 3). These are similar to the values previously reported for the human liver α -mannosidases A and B [2].

On SDS/PAGE under reducing conditions, the polypeptides of α -mannosidase B were resolved into bands with apparent molecular masses of 67, 59, 55 and 30 kDa (Figure 4).

Our results confirm the subunit structure reported for the human liver α -mannosidase B [2], but demonstrate a higher degree of microheterogeneity.

Automated Edman degradation of the 30 kDa band gave a partial amino acid sequence:

Ser-Pro-Ala-Leu-Thr-Ile-Glu-Asn-Glu-His-Ile-Arg-Ala-Thr-Phe-Asp-Pro-Asp-Thr

which was found to be identical with residues 551–569 of the sequence deduced from a cDNA [6] recently cloned and only tentatively identified as the α -mannosidase gene. Additionally, in the deduced amino acid sequence the presence of two arginine residues (at positions 547 and 548) might represent the point of proteolytic cleavage, with the subsequent removal of residues 549 and 550 to leave the mature N-terminus of the 30 kDa polypeptide, as already described in the processing of the β -hexosaminidase subunits by Mahuran et al. [16].

Confirmation of the identity of the human lysosomal α -mannosidase cDNA should now provide a secure basis for the analysis of mutations causing α -mannosidosis.

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