Comparison of the α -Mannosidases in Fibroblast Cultures from Patients with Mannosidosis and Mucopolipidosis II and from Controls

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(Received 1 February 1980)

The intracellular and extracellular acidic α -mannosidase in cultures of fibroblasts from mucopolipidosis-II patients has normal kinetics. The extracellular activity in cultures of cells from mannosidosis patients is normal, but a mutant enzyme is associated with the cell surface and intracellular fraction. The results support the involvement of membrane cycling and recognition markers in lysosomal enzyme localization.

Several forms of α -mannosidase with different subcellular localizations occur in mammalian tissues. The lysosomal forms (Carroll *et al.*, 1972; Chester *et al.*, 1975) have an acidic pH optimum, and the cytosol contains α -mannosidase activity with an optimum at neutral pH (Marsh & Gourlay, 1971). A third type of activity with an intermediate pH optimum (Snaith, 1973; Phillips *et al.*, 1974) has been purified from Golgi membranes (Tulsiani *et al.*, 1977). This intermediate α -mannosidase activity has also been detected in body fluids such as cerebrospinal fluid and urine (Chéron *et al.*, 1975), and in serum (Chéron *et al.*, 1975; Hirani *et al.*, 1977), where it is quantitatively the major form of α -mannosidase. Apart from their pH optima, the three forms can be distinguished by their characteristic responses to Co^{2+} and Zn^{2+} ions (Hirani *et al.*, 1977). The acidic form is activated by Zn^{2+} and inhibited by Co^{2+} . The intermediate form is activated by both ions, whereas the neutral form is activated by Co^{2+} and slightly inhibited by Zn^{2+} . As the Zn^{2+} activation is pH-dependent, the presence or absence of Zn^{2+} affects the apparent pH optimum.

The human lysosomal storage disease mucopolipidosis II is characterized by a multiple intracellular deficiency of lysosomal hydrolases, including acidic α -mannosidase, in cultured fibroblasts and lymphocytes, and by elevated activities of the same enzymes in extracellular fluids (Wiessman *et al.*, 1971). It has been suggested that the faulty distribution of hydrolases between the extracellular and lysosomal compartments in mucopolipidosis II is due to a defect in a common recognition marker on the enzymes (Hickman & Neufeld, 1972).

A specific deficiency of acidic α -mannosidase is associated with another human storage disease,

mannosidosis (Öckerman, 1967), and a mutation in the structural gene has been proposed as the primary defect in the disease (Beaudet & Nichols, 1976). A recognition defect, similar to that in mucopolipidosis II, but affecting only α -mannosidase, has also been postulated (Hultberg & Masson, 1977).

In the present study some characteristics of the intra- and extra-cellular α -mannosidase activities in fibroblast cultures from patients with human mannosidosis and mucopolipidosis II are compared with the corresponding activity in normal controls. In addition, we have attempted to separate that fraction of the activity that is associated with the external surface of the fibroblasts to ascertain whether it originates by re-adsorption of exocytosed enzyme from the medium or by exteriorization of intracellular activity.

Materials and Methods

Cell culture

Fibroblasts were cultured from three normal adults, a patient with mannosidosis and a patient with mucopolipidosis II, in Ham's F10 medium (Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 10% (v/v) pH-inactivated foetal-calf serum (Flow Laboratories), penicillin (100 units/ml of medium) and streptomycin (0.1 mg/ml of medium) (both from Gist-Brocades, Delft, The Netherlands). Fresh medium was left in contact with the resuspended cells from confluent cultures of fibroblasts for 5 days (1.5 ml of medium/ 3×10^5 – 5×10^5 cells), after which time it was poured off, centrifuged (90g for 10 min) and the cell-free supernatant assayed for enzyme activity. This represents the extracellular activity. The pericellular fraction was

prepared by 10 min treatment with 0.25% (w/v) trypsin (ICN Pharmaceuticals, Cleveland, OH, U.S.A.), followed by 1% soya-bean trypsin inhibitor (Worthington, Freehold, NJ, U.S.A.) in saline (0.9% NaCl) and centrifugation (140g for 10 min). This supernatant represented the pericellular activity and the pellet the intracellular fraction. Cell homogenates were prepared by five cycles of freezing and thawing by using liquid nitrogen. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme assays

α -Mannosidase activities were assayed by incubation of a 10 μ l sample with 30 μ l of 4-methylumbelliferyl α -D-mannopyranoside in Sorensen's citrate buffer (0.1 M-citric acid in 0.2 M-NaOH adjusted to the appropriate pH with 0.1 M-HCl or -NaOH) for 3 h at 37°C. The final substrate concentration was 1 mM. The reaction was stopped by the addition of 500 μ l of 0.5 M-Na₂CO₃ adjusted to pH 10.7 with NaOH, and fluorescence was read in a Perkin-Elmer fluorimeter (emission wavelength 448 nm, excitation at 365 nm). Kinetic studies were performed with final substrate concentrations between 0.5 and 20 mM. The influence of pH was tested between pH 2.6 and 7.2 at a protein concentration of 2.5 μ g/10 μ l. After inactivation at pH 10, the foetal-calf serum contained less than 1% of the original acidic α -mannosidase activity, which was slightly re-activated by incubation at 37°C. All extracellular activities were therefore corrected for the residual calf α -mannosidase activity present in the serum. Where indicated, ZnSO₄ or CoCl₂ was included in the reaction mixture at 1 mM final concentration, which did not influence the pH of the assay mixture.

Results and Discussion

The activity-pH profiles for the intra- and extra-cellular α -D-mannosidase in control fibroblasts showed the expected acidic optimum, pH 4.7, corresponding to acidic α -D-mannosidase and a shoulder of activity between pH 6.0 and 7.0 corresponding to neutral α -D-mannosidase. The pH optimum of the neutral α -D-mannosidase, pH 6.3–6.4, was clearly seen in the intracellular activities from cells from mannosidosis and mucopolipidosis-II patients, in which there was a deficiency of acidic α -D-mannosidase. Table 1 shows the relative intra- and extra-cellular activities of acidic and neutral α -D-mannosidase in the strains from mannosidosis and mucopolipidosis-II patients and in three control strains. The activity and distribution of the neutral enzyme are clearly not affected in either of the mutant strains, both of which are characterized by a profound intracellular deficiency

Table 1. *Acidic and neutral α -mannosidase activities in fibroblast cultures from three controls and from a mannosidosis and a mucopolipidosis-II patient*

The activities are the means of four cultures of each cell strain, with the ranges in parentheses, related to the intracellular protein.

	α -Mannosidase (munits/mg of protein)			
	Acidic		Neutral	
	Intra-	Extra-	Intra-	Extra-
Control 1	48 (44–51)	30 (27–34)	22 (21–24)	31 (27–33)
Control 2	62 (56–65)	14 (13–15)	30 (29–31)	15 (14–16)
Control 3	37 (35–40)	19 (17–21)	25 (24–25)	17 (15–19)
Mannosidosis	2 (1.7–2.3)	11 (8–13)	21 (20–23)	20 (19–21)
Mucopolipidosis II	2 (2.0–2.3)	85 (72–95)	11 (10–12)	15 (12–20)

of acidic α -D-mannosidase. Although the extra-cellular acidic α -D-mannosidase of the mucopolipidosis-II-patient cells is high, as expected for a defect in the localization process, that for the mannosidosis cells is relatively low. Nevertheless there is a significant amount of the acidic activity in the medium from both of these cells types, despite the intracellular deficiency.

The intra- and extra-cellular acidic α -D-mannosidase activities in normal and mucopolipidosis-II-patient cells all have similar K_m values and responses to Co²⁺ and Zn²⁺ (Table 2). This suggests that the defect in processing of lysosomal hydrolases in mucopolipidosis II does not affect the kinetic properties of acidic α -D-mannosidase. In contrast, the intracellular acidic activity from cultures of fibroblasts from a patient with mannosidosis was different from the extracellular activity, which appeared to be normal. The higher K_m value and activation by Co²⁺ of the intracellular activity were consistent with it being a mutant enzyme. A difference between the intra- and extra-cellular acidic α -D-mannosidase activities in mannosidosis had previously been reported by Hultberg & Masson (1977), who interpreted their observation as a specific defect in the cellular resorption of acidic α -D-mannosidase in this disorder. Therefore it was decided to examine the nature of the pericellular enzyme that could be released from the surface of mannosidosis cells by treatment with trypsin. This enzyme fraction could represent exocytosed intracellular enzyme that has not been released into the medium, or re-adsorbed extracellular enzyme that for some reason was not endocytosed. This enzyme fraction was found to resemble the residual intra-

Table 2. *Effect of Zn²⁺, Co²⁺ and substrate concentration on acidic α -mannosidase*

Numbers in parentheses indicate numbers of determinations. Inhibition or activation in the presence of Zn²⁺ or Co²⁺ is expressed as the activity in the presence of the ion as a percentage of that activity in its absence.

	Intracellular			Extracellular		
	K_m (mM)	Zn ²⁺ activation at pH 4.0 (%)	Co ²⁺ inhibition or activation at pH 4.7 (%)	K_m (mM)	Zn ²⁺ activation at pH 4.0 (%)	Co ²⁺ inhibition or activation at pH 4.7 (%)
Control	0.64	271 (n = 2)	80	0.80 (n = 4)	219	66
Mannosidosis	6.42 (n = 3)	327 (n = 2)	276 (n = 4)	1.27 (n = 3)	259	37 (n = 2)
Mucopolipidosis II	0.66 (n = 2)	254	63	0.80 (n = 3)	286 (n = 2)	51

cellular activity in being stimulated 2–2.5-fold by Co²⁺ and having a high apparent K_m value (7.48 mM). Recent preliminary results (A. d'Azzo & D. J. J. Halley, unpublished work) indicate also that there is no intercellular transport of secreted α -D-mannosidase from cells of a mannosidosis patient to those of a mucopolipidosis-II patient in co-cultivation studies. Thus we may suppose that two forms of acid α -mannosidase are generated by the cell strain from the mannosidosis patient. A small amount of activity with abnormal kinetic properties remains in the cell and may be found exteriorized on the cell surface. On the other hand, the majority of the enzyme that is synthesized has normal kinetics, but is secreted into the medium and apparently lacks the essential properties for binding to the membrane and uptake into the cells.

These results provide evidence for membrane cycling in the localization of lysosomal enzymes (Lloyd, 1977) and for recognition between cell receptors and markers on lysosomal enzymes (Hickman *et al.*, 1974). The clear difference between the extra- and intra-cellular acidic α -mannosidase in mannosidosis make this disorder a valuable model for studying the mechanism of localization of lysosomal enzymes.

We thank Dr. A. D. Patrick, Dr. A. Boué and Dr. M. F. Niermeijer for kindly providing us with the patients' fibroblasts. L. J. B. was the recipient of a Medical Research Council research studentship. This work was supported in part by the Netherlands Organization for the Advancement of Research (Z.W.O.) and by the British Council.

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