EVIDENCE THAT THE MUTANT ENZYME IN FIBROBLASTS OF A PATIENT WITH MANNOSIDOSIS DOES NOT CROSSREACT WITH ANTISERUM RAISED AGAINST NORMAL ACIDIC α-D-MANNOSIDASE

Lynda J. BURDITT, Kokila A. CHOTAI and Bryan G. WINCHESTER
Department of Biochemistry, Queen Elizabeth College, University of London, Campden Hill Road, London W8 7AH, England

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1. Introduction

Mannosidosis is a lysosomal storage disease of humans [1,2] and Angus cattle, [3,4] resulting from a deficiency of lysosomal acidic α-D-mannosidase (EC 3.2.1.24). It has been suggested on the basis of its altered stability and kinetic properties that a mutant enzyme, resulting from a mutation in a structural gene, accounts for the residual acidic α-D-mannosidase in human [5–7] and bovine [8] mannosidosis. The mutant enzyme in bovine mannosidosis crossreacts with antiserum raised against normal bovine acidic α-D-mannosidase and accounts for all the crossreacting material present in bovine mannosidosis [9]. Antiserum raised against pig kidney acidic α-D-mannosidase crossreacts with the acidic α-D-mannosidase in normal human fibroblasts and in other human and bovine tissues [10]. Crossreacting material has also been detected by radio-immunological techniques in fibroblasts from two patients with mannosidosis using this antiserum [11]. However, we present evidence in this paper that the mutant enzyme in another patient with mannosidosis does not crossreact with antiserum raised against normal human liver acidic α-D-mannosidase. Further no enzymically inactive crossreacting material was detected.

2. Materials and methods

2.1. *Tissues*

Post-mortem human liver was stored at −20°C until required. Skin fibroblasts from a patient with mannosidosis (case 1 reference 12) were kindly supplied by Dr A. D. Patrick (Institute of Child Health, London). Control fibroblasts were obtained by skin punch biopsy from normal donors. Fibroblasts were grown in 25 cm^2 Falcon flasks containing basal medium (Eagle) dissolved in Earle's balanced salt solution (Flow Laboratories Ltd, Irvine, Scotland) with addition of $NaHCO_3$ (2.2 g/l), glutamine (2 mM) and foetal calf serum (15% v/v) (Gibco-Biocult, Paisley, Scotland). The flasks were incubated at 37°C in CO_2:air (5:95, v/v) atmosphere. Cells were harvested using a solution of trypsin (0.1%, w/v) (Wellcome Laboratories Ltd, Beckenham) in Hanks balanced salt solution (Flow). After 3 washes with phosphate buffered saline (Flow) and centrifugation, the cells were sonicated for two 15 s pulses at 20 kHz in an MSE Ultrasonic disintegrator. The supernatant after further centrifugation was used. Cells were counted using a haemocytometer.

2.2. *α-D-mannosidase assay*

α-D-mannosidase was assayed using the fluorigenic substrate 4-methylumbelliferyl α-D-mannopyranoside (Koch-Light Laboratories Ltd, Colnbrook, Bucks) as in [2] except that the substrate concentration was increased to 5 mM in the reaction mixture. One unit of activity is the amount of activity that transforms 1 μmol substrate/min under these conditions.

2.3. *Preparation of acidic α-D-mannosidases A and B*

α-D-mannosidases A and B were prepared from normal human liver and fibroblasts by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52)

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[2]. Neutral α-D-mannosidase was removed from the liver extract by chromatography on concanavalin A–Sepharose (Pharmacia (GB) Ltd, London) prior to ion-exchange chromatography [13].

2.4. *Serological experiments*

Antiserum was raised in a rabbit against partially purified (0.3 U/mg protein) human liver acidic α-D-mannosidase [13,14]. Rabbit acidic α-D-mannosidase was removed from the antiserum by chromatography on concanavalin A–Sepharose. The glycosidases bound to the column whereas the immunoglobulin fraction was unretarded. Immunodiffusion and immunoprecipitation experiments were carried out as in [14]. Protein was determined by the Folin method [15] using bovine serum albumin as the standard.

3. Results

3.1. *Immunoprecipitation of the α-D-mannosidase in normal human fibroblasts and in fibroblasts of a patient with mannosidosis*

The antiserum raised against the human liver acidic α-D-mannosidase precipitated all the acidic α-D-mannosidase from an extract of a normal human liver (fig.1). This indicated that both the major forms of acidic α-D-mannosidase, A and B, separable by chromatography on DEAE-cellulose were precipitated. The neutral α-D-mannosidase present did not crossreact. The majority of the acidic α-D-mannosidase in normal fibroblasts was also precipitated by the antiserum but it was never possible to precipitate all the activity. The proportion of acidic activity remaining in solution varied from 10–40%. To investigate whether the intermediate or neutral α-D-mannosidases present in fibroblasts crossreacted with the antiserum, the α-D-mannosidase activity was also assayed at pH 5.5. The immunoprecipitation curve shows that ~50% activity at this pH is precipitated. However the acidic α-D-mannosidase precipitated by the antiserum has appreciable activity at pH 5.5 and this would account for the activity at this pH precipitated by the antiserum. It is concluded that the intermediate or neutral α-D-mannosidases do not crossreact with the antiserum.

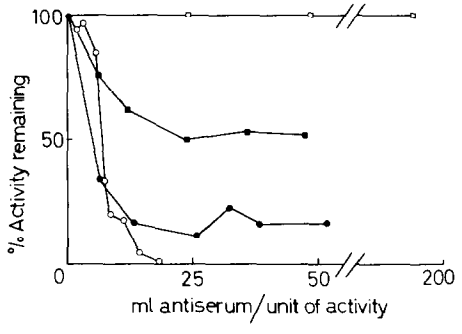

Fig.1. Immunoprecipitation of α-D-mannosidase in liver and fibroblasts. The enzyme preparation (50 μl) and antiserum (1–10 μl) were mixed and made up to final vol. 150 μl with phosphate-buffered saline, pH 7.4. The supernatants obtained after centrifugation of the mixtures were assayed for α-D-mannosidase at pH 4.0. (○) Liver (0.7 mU); (●) normal fibroblasts (0.155 mU); (□) mannosidosis fibroblasts (0.041 mU); (■) normal fibroblasts assayed at pH 5.5 (0.169 mU).

The residual acidic α-D-mannosidase in the fibroblasts of a patient with mannosidosis was also not precipitated by the antiserum. This showed either that the mutant enzyme did not crossreact with the antiserum or that its avidity for the antibodies was very much lower than that of the normal enzyme. Immunoprecipitation was also carried out with mixtures of various proportions of material from normal and mannosidosis fibroblasts. The presence of material from fibroblasts of the patient with mannosidosis did not alter the concentration of antiserum required to precipitate the normal acidic α-D-mannosidase. This suggests either that enzymically inactive crossreacting material is absent in mannosidosis or that it is present in low concentrations and/or has a low avidity for the antibodies.

3.2. *Immunodiffusion*

When fibroblast acidic α-D-mannosidase A and B, prepared by chromatography on DEAE-cellulose, were placed in adjacent wells a continuous active immunoprecipitin band was observed (fig.2a). There was no evidence of spur formation. As both forms react with the antiserum, it is unlikely that a specific form of acidic α-D-mannosidase accounts for the activity that was not precipitated in the immunoprecipitation experiments. The active precipitin line was also continuous with those obtained for a whole fibroblast extract, a liver extract and α-D-mannosidase A and B prepared from liver Therefore the acidic α-D-

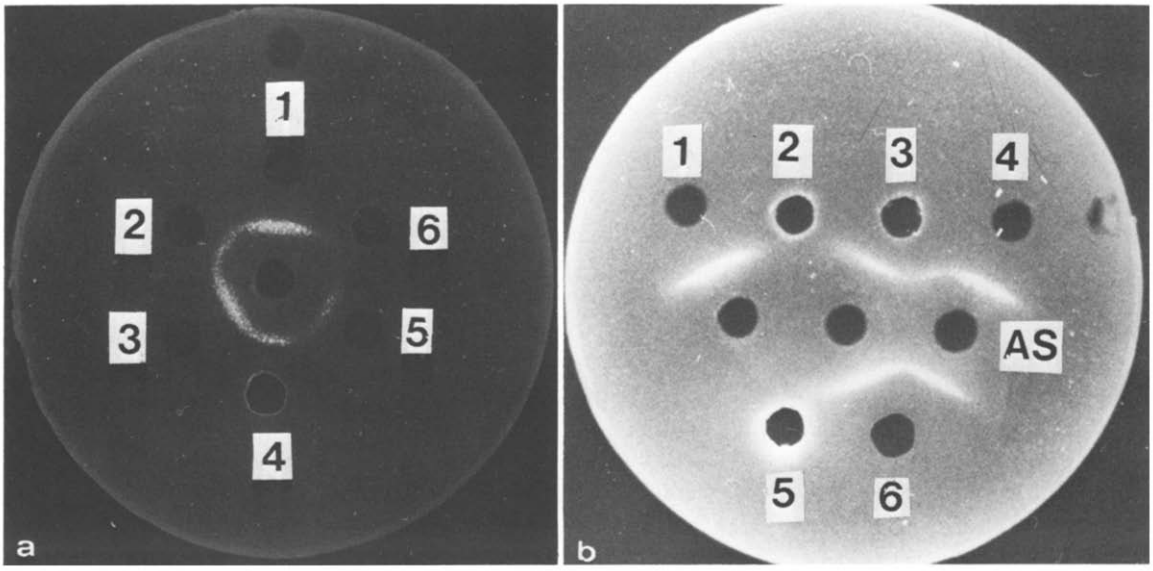

Fig.2. Immunodiffusion of liver and fibroblast acidic α-D-mannosidase against anti (α-D-mannosidase)-serum (AS). The immunoprecipitin lines were detected enzymically and the photographs are reproduced negatively. (a) 1, normal liver; 2, 3, liver acidic α-D-mannosidase A and B, respectively; 4, normal fibroblasts; 5, 6, normal fibroblast acidic α-D-mannosidase A and B, respectively. (b) 1, 4, 6 normal human liver; 2, mannosidosis fibroblasts; 3, normal fibroblasts; 5, normal bovine liver.

mannosidase in normal human fibroblasts is immunologically identical to that in liver.

No enzymically active precipitin line was observed with an extract of the fibroblasts from the patient with mannosidosis. Further no enzymically inactive crossreacting material was detected when the immunodiffusion plate was post-stained for protein. Protein bands corresponding to the enzymic activity were detected in normal liver and fibroblasts. Another crossreacting component not coincident with the enzyme was observed in human liver. It has been detected in human liver with this antiserum and is probably co-purified with the enzyme [13,14]. Either it does not occur in fibroblasts or its concentration is too low for detection. The antiserum did not crossreact with normal bovine liver acidic α-D-mannosidase.

4. Discussion

The lack of crossreaction between the antiserum raised against the normal enzyme and the mutant enzyme in the fibroblasts from a patient with mannosidosis suggests that the mutation affects the antigenic sites as well as the stability and kinetic properties of α-D-mannosidase. In addition no enzymically inactive crossreacting material was detected by immunoprecipitation or immunodiffusion. In contrast evidence has been found by a radio-immunological technique, for the occurrence in fibroblasts from another patient with mannosidosis of material that crossreacts with anti-(pig acidic α-D-mannosidase) serum [9,10]. This antiserum has a wide specificity [10], crossreacting with the normal bovine and human enzymes, whereas the anti-(human α-D-mannosidase) serum does not crossreact with the bovine enzyme. Therefore it is possible that the difference in specificity or avidity of the antisera might explain this apparent discrepancy. However, if the crossreacting material was present in mannosidosis-fibroblasts in comparable amounts with comparable avidity to those of the normal enzyme in normal cells, as has been suggested [11], it should have been detected with our antiserum. An alternative explanation is that there are variants of mannosidosis. The residual acidic α-D-mannosidase in the fibroblasts of the patient with mannosidosis was thermolabile

but the K_m value was only 1.3 mM compared with 0.8–0.9 mM for controls (L.J.B., B.G.W., unpublished results). A 25-fold increase in the K_m value has been reported for the mutant enzyme in other cases of mannosidosis [6,16]. However the K_m value for the activity in one of the patients in which crossreacting material was detected was not significantly different from that of the control [17]. It has been claimed in other cases that the residual α-D-mannosidase is activated 6–10-fold by Zn^{2+} [18] or that the value of K_m is decreased by Co^{2+} [7]. In our case the mutant enzyme is only activated by 20% by Zn^{2+} over a wide range of concentration and is inhibited by Co^{2+} (unpublished results). Therefore it is possible that there are variant forms of mannosidosis, which can be distinguished biochemically by differences in the kinetic properties and possibly immunological properties of the mutant enzyme.

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