EVIDENCE FOR MATERIAL FROM MANNOSIDOSIS FIBROBLASTS CROSSREACTING WITH ANTI-ACIDIC α-MANNOSIDASE ANTIBODIES

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1. Introduction

Mannosidosis is a lysosomal storage disease [1] caused by the deficiency of α-mannosidase (EC 3.2.1.24) forms A and B with an acidic pH-optimum [2–4]. The residual α-mannosidase activity present in mannosidosis tissues and cultured cells is structurally and genetically different, it can be activated by Co^{2+} and is heat labile [2–6]. At extremely high substrate concentrations a residual acidic α-mannosidase activity with altered K_m and thermal stability becomes detectable in mannosidosis fibroblasts [7]. This was interpreted as due to a structurally altered enzyme formed in mannosidosis instead of the normal acidic α-mannosidase. We give here radioimmunological evidence for the existence of material in mannosidosis fibroblasts that reacts with antibodies specifically binding acidic α-mannosidases from different sources.

2. Materials and methods

Carrier free $Na^{125}I$ was purchased from Amersham-Buchler (Braunschweig) and bovine serum albumin (> 99%) from Serva (Heidelberg). Conditions for culturing cells [8,9] preparation of cell homogenate [10] assay of α-mannosidase activity [10] and protein concentration [11] were as referenced.

Pure acidic α-mannosidase was prepared from pig kidneys and rabbit antibodies against this pure enzyme raised essentially according to known procedures [12,13]. ^{125}I-Radioactivity was measured in a LKB-Wallac 80 000 γ-counter or a Packard Modumatic VI Auto Gamma Model 5360.

Abbreviations: BSA Bovine serum albumin, PBS Phosphate buffered saline. Enzyme: α-Mannosidase, α-D-mannosidase mannohydrolase (EC 3.2.1.24)

2.1. *Iodination of α-mannosidase*

Iodination was performed essentially according to David and Reisfeld [14]. 100 μg of pure α-mannosidase in 0.1 ml 0.01 M sodium phosphate buffer, pH 7.0, containing 0.14 M NaCl (PBS), 5 μl (= 0.5 mCi) carrier free $Na^{125}I$, about 20 μg lactoperoxidase coupled to Sepharose 4 B [14] (kind gift of Dr von Figura, Münster) and 5 μl of a 1 mM solution of H_2O_2 were shaken for 5 min at room temperature. 5 μl of 0.62 mM unlabelled potassium iodide were added and shaking continued for another 10 min. After this time 5 μl 3.5 M NaN_3 and 5 μl PBS containing 2% (w/v) bovine serum albumin (BSA) were added and the mixture submitted to Sephadex G-25 gelfiltration (fig.1).

0.1 ml portions of the pooled material were further purified by polyacrylamide disc-gel electrophoresis (fig.2).

2.2. *Immunoprecipitation*

Solutions used for immunoprecipitation were diluted with 0.01 M sodium phosphate buffer pH 7.0, 0.14 M NaCl, 2% (w/v) bovine serum albumin (PBS/2% BSA). Suitable amounts of antibody dilutions and of antigen solution were incubated for 2 h at 37°C and overnight at 4°C and then centrifuged at 2000 × *g* for 20 min. α-Mannosidase was assayed from an aliquot of the supernatant. For the deter-

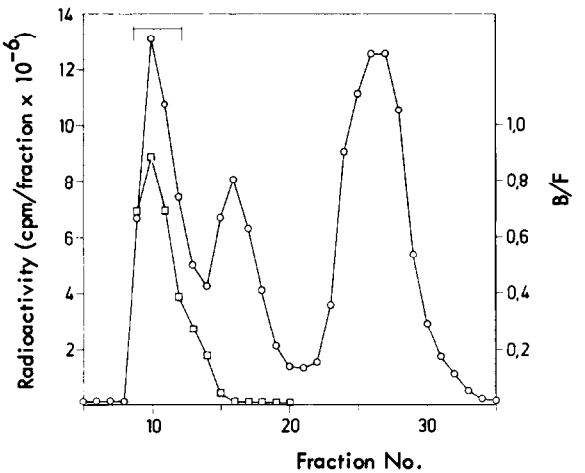

Fig.1. Gel filtration of pig kidney ^{125}I-α-mannosidase. About 150 μl of reaction mixture were applied to a Sephadex G-25 column (0.6 × 37 cm) and eluted with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.14 M NaCl and 0.5% bovine serum albumin (PBS/0.5% BSA). Flow rate was 15 ml/h, fraction vol. 0.5 ml. (○——○) radioactivity, (□——□) ratio of bound to free radioactivity (B/F) after incubation of 5 μl eluate with 1 μl antibody solution and 90 μl of PBS/2% BSA. The horizontal bar indicates the fractions pooled for further purification.

mination of the ratio of bound to free labelled antigen (B/F) the supernatant was carefully removed from the precipitate, the precipitate once washed with PBS/2% BSA, again centrifuged as above and the supernatant once more removed and the radioactivity measured in the combined supernatants and the residual precipitate. In blanks antigen solutions were replaced by PBS/2% BSA, antibody solutions by a suitable mixture of rabbit serum and the dilution buffer.

3. Results

3.1. *Preparation of ^{125}I-labelled pig kidney α-mannosidase*

α-Mannosidase with an optimum pH of 4.2, purified from pig kidney to homogeneity was labelled with ^{125}I and the labelled enzyme purified by gel filtration and disc-gel electrophoresis (fig.1 and 2). After gel filtration fractions containing about 21% of the radioactivity and 82% of the initial α-mannosidase activity were pooled; about 45–50% of this material was bound by anti-α-mannosidase antibody.

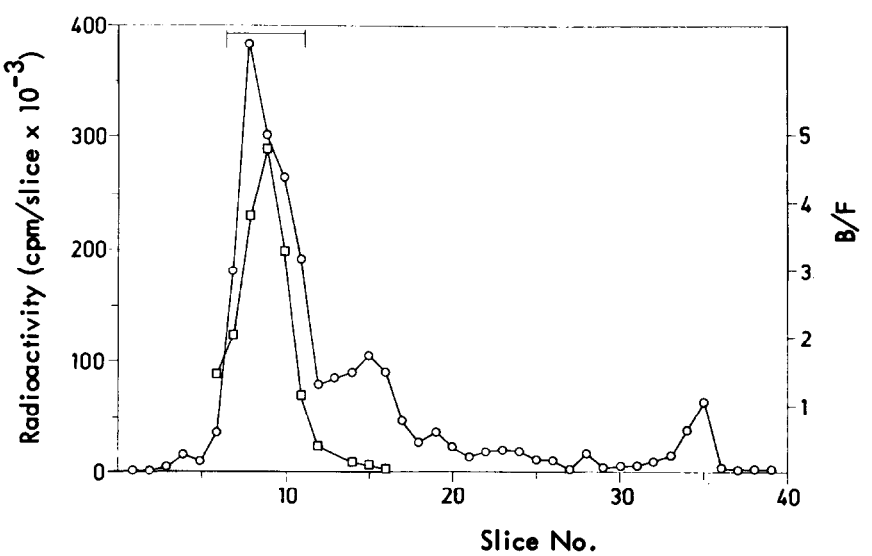

Fig.2. Polyacrylamide disc-gel electrophoresis of pig kidney ^{125}I-α-mannosidase. 100 μl of pooled eluate from the gel-filtration step were run on a 6.16% polyacrylamide gel, system 1 according to Maurer [15]. The gel was cut into 2 mm slices, these were counted for radioactivity (○——○), three times eluted with 0.2 ml PBS/2% BSA. 10 μl of the extracts were incubated with 1 μl antibody solution and 100 μl PBS/2% BSA. (□——□) Ratio of bound to free radioactivity (B/F). The horizontal bar indicates the slices extracted for further use.

Due to poor extractability of the gel slices – about 60% of total radioactivity could be extracted in 3 changes – only 51% of total α-mannosidase together with 34% of the radioactivity applied to the gels could be recovered. This material exhibited good antigenicity: about 80% of its radioactivity was bound by antibody. Slow decomposition of the labelled enzyme occurred even on storage at –20°C, leading to the formation of material moving faster in disc-gel electrophoresis and to a decrease in binding by antibody.

3.2. *Determination of crossreacting material in fibroblast homogenates*

In a first set of experiments ratios of antibody to antigen solutions that gave both 50–80% precipitation of labelled pig kidney α-mannosidase and complete binding of acidic α-mannosidase from human control fibroblasts were determined. The quantity of antibody solution so determined was first incubated with a set of cell homogenate dilutions from control and mannosidosis fibroblasts then centrifuged. The requisite quantity of labelled pig kidney α-mannosidase was added to an aliquot of the supernatant and after a second incubation the ratio of bound to free radioactivity was measured in these assays. It decreased with the amount of cell protein added to the first incubation mixture. Similar results were obtained with cell homogenates of control fibroblasts with an intracellular α-mannosidase activity (at pH 3.9) of about 3.76 mU/mg cell protein and of mannosidosis cells from two different lines which exhibited a mean residual activity of 0.36 mU/mg cell protein (fig.3).

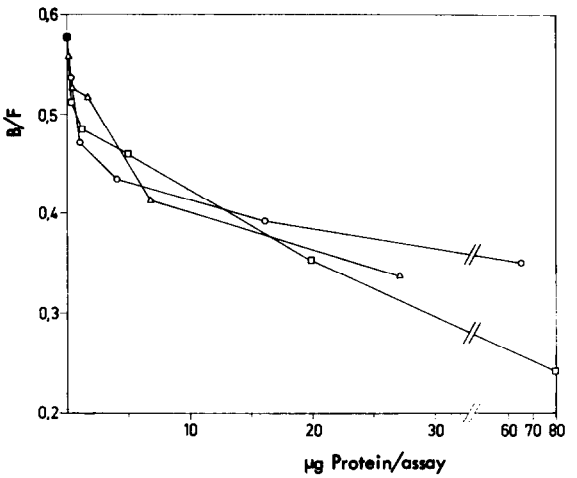

Fig.3. Radioimmunoassay of cell homogenate material crossreacting with acidic α-mannosidase. 60 µl antibody solution diluted 1 : 64 were preincubated as described under Materials and methods with 60 µl cell homogenate of different dilutions and centrifuged. 100 µl of supernatant were again incubated with 10 µl of pig kidney ^{125}I-α-mannosidase diluted 1 : 16 and the ratio of bound to free radioactivity (B/F) was measured as described in the text. (○——○) Control cells, (□——□ and △——△) two different cell lines derived from mannosidosis patients, (■) Blank. Each point is the mean of six determinations.

4. Discussion

Antiserum raised in rabbits immunized with pure acidic α-mannosidase from pig kidneys shows crossreactivity of different avidity with acidic α-mannosidases from various sources, including cultured human skin fibroblasts (G. Mersmann and E. Buddecke, unpublished results). Preincubation of this antibody solution with cell homogenates from control and mannosidosis fibroblasts, centrifugation and subsequent reaction of the supernatant with an excess of ^{125}I-labelled pig kidney α-mannosidase demonstrate the existence of enzymatically inactive material immunologically crossreacting with acidic α-mannosidase. This procedure of preincubation with the unlabelled antigen, separation of the formed antigen–antibody complex and determination of the residual antibody concentration with excess labelled antigen gave better results than a method of incubation with labelled pig kidney α-mannosidase and unlabelled human antigen at the same time. This is presumably due to the great differences in avidity of the antibodies against α-mannosidase from pig kidney and of human origin. A strictly quantitative determination of the concentration of crossreacting material in mannosidosis cells was not possible. The results of fig.3 may, however, allow one to conclude that the amount of crossreacting material is of the same order of magnitude in control and mannosidosis cells assuming similar avidities for the normal and genetically altered proteins. The possibility that the crossreacting material in mannosidosis cells is the neutral form C of α-mannosidase is excluded as this genetically

different enzyme form is not bound to antibodies against the acidic forms of α-mannosidase [16].

The results of our immunological experiments confirm and extend the conclusion of Beaudet and Nichols [7], drawn from the detection in mannosidosis cells of α-mannosidase activity with an extremely high K_m, that mannosidosis is caused by a defect of a structural gene leading to the formation of a protein with lower though not totally lacking affinity for its substrate, with altered thermal stability, but with close immunological similarities to normal acidic α-mannosidase. These findings for mannosidosis are in accordance with those for other lysosomal storage diseases in which defects of structural genes are also thought to be responsible (for references see [13]).

As enzyme replacement has recently been shown to be possible in cultured mannosidosis fibroblasts [10] the presence in these cells of material cross-reacting immunologically with α-mannosidases that can correct the defect is of importance as this increases the possibility of solving the immunological problems of enzyme replacement therapy in mannosidosis patients.

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