GENETIC HETEROGENEITY OF α-GALACTOSIDASE IN FABRY'S DISEASE

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1. Introduction

Fabry's disease is an X-linked disorder characterized by the abnormal accumulation of a neutral glycolipid, trihexosylceramide (galactosylgalactosylglucosylceramide) [1]. The clinical manifestations include a vascular rash of the skin, febrile crises, and severe pain in the extremities. The deficient enzyme is the one which cleaves the glycosidic bond between the terminal and pre-terminal galactose residues of trihexosylceramide [2]. A deficiency of α-galactosidase (EC 3.2.1.22), assayed with the synthetic substrates p-nitrophenyl α-galactopyranoside and 4-methylumbelliferyl α-galactopyranoside, has been found in leukocytes [3] and skin fibroblasts [4, 5] of patients. Heterozygotes have two clonal populations of fibroblasts, one with α-galactosidase activity similar to controls and the other with activity in the range of patients [4].

The specificity of the assay for α-galactosidase, based on the use of synthetic substrates, has been questioned [6, 7] because it was thought that the terminal glycosidic bond of the natural substrate had a β-anomeric configuration, but recent studies have shown the terminal galactose of trihexosylceramide to be an α-anomer [8–11]. However, the enzymatic assay based on the use of synthetic substrates might measure the activity of more than one α-galactosidase and, indeed, two forms of the enzyme have been partially purified and characterized from human kidney [12]. They can be differentiated as a thermolabile form (A) and a thermostable form (B). The A form has a more acidic pl (4.6) than the B form (pl 4.9) and migrates faster towards the anode on Cellogel electrophoresis. Only the A form is deficient in most patients affected with Fabry's disease, as shown by this and other studies [13–15]. However, in one of the patients presented here, both forms of α-galactosidase and a residual activity towards the natural substrate, trihexosylceramide, were present. This finding can be explained by heterogeneity of the mutations affecting the structural locus coding for α-galactosidase, which has recently been shown by means of somatic cell hybrids to be X-linked in man [16].

2. Materials and methods

2.1. Cell culture

Five unrelated patients affected with Fabry's disease were studied (table 1), two of whom (no. 4 and no. 5) were recently described as clinical variants of the disease since they had no skin lesions [7]. Fibroblast cultures were initiated from skin biopsies of patients and controls and cells were harvested and sonicated as previously described [4].

2.2. Assay of α-galactosidase

The enzymatic activity of α-galactosidase was assayed in an incubation mixture containing 625 nmoles of 4-methylumbelliferyl α-D-galactopyranoside (Koch-Light), 10 μmoles of acetate buffer at pH 5.0, and 10 μl of cell sonicate, incubated in a volume of 65 μl for 30 min at 37°. The reaction was stopped by adding
Table 1

Properties of α-galactosidase from patients and controls as assayed with the synthetic substrate, 4-methylumbelliferyl-α-galactoside.

<table>
<thead>
<tr>
<th>Fibroblast line</th>
<th>Specific activity (units/mg protein)</th>
<th>$K_m$ value (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) M.M.</td>
<td>4.1</td>
<td>26.8</td>
</tr>
<tr>
<td>2) J.L.</td>
<td>7.1</td>
<td>16.5</td>
</tr>
<tr>
<td>3) F.L.</td>
<td>8.8</td>
<td>15.8</td>
</tr>
<tr>
<td>4) A.H.</td>
<td>3.3</td>
<td>21.6</td>
</tr>
<tr>
<td>5) R.C.</td>
<td>10.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Control average ± S.D. (Number of controls)</td>
<td>29.2 ± 5.3 (5)</td>
<td>2.1 ± 0.7 (6)</td>
</tr>
</tbody>
</table>

0.1 M Tris to a final volume of 5 ml. Appropriate dilutions were read in a Turner fluorometer equipped with 110.811 primary filters and 110.816 and 831 secondary filters and standardized with 4-methylumbelliferone. Protein content was measured in duplicates [17], and the specific activity was expressed as nmoles of substrate hydrolyzed/mg of protein/hr at 37°C and pH 5.0. The enzymatic activity was linear with protein concentration.

2.3. Assay of β-galactosidase

The incubation mixture containing 20 μmoles of acetate buffer (pH 4.0), 3 μmoles of NaCl, 0.1 μmoles of 4-methylumbelliferyl β-D-galactopyranoside (Pierce) and 10 μl of cell sonicate in a final volume of 0.25 ml was incubated for 15 min at 37°C. The reaction was stopped by adding 0.1 M Tris to a volume of 10 ml or more, and readings were taken as described above.

2.4. Purification and radioactive labelling of trihexosylceramide

The trihexosylceramide was isolated from an acetone powder of 1 kg of human kidney by the method of Martensson [18] and further purified by preparative thin-layer chromatography on silica gel G in: i) chloroform–methanol–NH₄OH (60:30:8), to separate glycolipids from residual phospholipids; and ii) chloroform–methanol–H₂O (30:30:4), to separate trihexosylceramide from globoside.

The purified trihexosylceramide migrated as a single spot on both thin-layer chromatography systems described above, and was free of contaminating phospholipids as determined by the molybdenum test [19]. The molar ratio of galactose to glucose liberated by enzymatic hydrolysis [10] agreed with that expected (2:1), and 92% of the expected total sugar residues were recovered after acid hydrolysis [20].

About 20 mg of this purified trihexosylceramide were labeled in the terminal galactose residue by oxidation with galactose oxidase (125 units, Worthington) followed by reduction of 25 μCi of $^3$H-NaBH₄ (524 mCi/mmole, Amersham) [20]. After dilution with 20 mg of unlabeled trihexosylceramide, 95% of the radioactivity migrated as the marker on thin-layer chromatography. The specific activity of this $^3$H-trihexosylceramide was 28 μCi/μmole. In order to show that the only labeled sugar residue in the molecule was the terminal galactose, the $^3$H-trihexosylceramide was incubated with α-galactosidase purified from fig latex and with β-galactosidase purified from jack bean [10]. The former glycosidase cleaves the terminal bond of trihexosylceramide (Gal $^1α$-Gal), while the latter cleaves the preterminal bond (Gal $^1β$4-Glc). Aliquots of 10 μmole of $[^3]$H-trihexosylceramide were incubated, respectively, with fig α-galactosidase alone or sequentially with both fig α-galactosidase and jack bean β-galactosidase [10]. No further radioactivity was liberated when the $[^3]$H-trihexosylceramide treated with α-galactosidase was subjected to a second incubation with β-galactosidase. Following the same procedure, a second batch of $[^3]$H-trihexosylceramide with high specific activity (240 μCi/μmole) was prepared.

2.5. Assay of trihexosylceramide α-galactosidase

The activity of trihexosylceramide α-galactosidase was assayed with a modification of the procedure described by Brady et al. [2]. The reaction mixture (0.1 ml) contained 20 nmoles of radioactive substrate, suspended in 10 μl of a solution of 2% sodium cholate (w/v), 10 μmoles of potassium acetate (pH 5.0), 1 mg of human serum albumin and enzyme. After 4 hr of incubation at 37°C, the reaction was stopped by adding in this order: 0.9 ml of cold H₂O, 0.1 ml of 10% bovine serum albumin and 0.1 ml of 100% trichloroacetic acid. After mixing and centrifugation, the pellet was resuspended in 1 ml of 10% cold trichloroacetic acid and treated as before. To the combined supernatants 1 ml of anhydrous ether was added and, after mixing and centrifugation, an aliquot of the lower
phase was counted in a scintillation vial with Bray's solution. At least 92% of the galactose was recovered at the end of this procedure.

2.6. Partial purification of α-galactosidase

The enzyme from fibroblast cultures was partially purified by ethanol precipitation. Cells from 10 to 20 petris (6.5 cm diameter) were harvested with a rubber policeman after one month in culture (see Results), and the pellet was resuspended in 2 to 4 ml of 0.01 M acetate buffer, pH 4.5, containing 0.4% Na deoxycholate and sonicated. After centrifugation for 15 min at 25,000 g, 95% ethanol was added to the supernatant with stirring at -10° to a final conc. of 61%. After centrifugation the pellet was resuspended in 0.5 ml of 1 mM citrate-phosphate buffer, pH 7.0, and spun again. More than 70% of the initial activity was recovered in the clear supernatant with a 4-fold purification of α-galactosidase.

3. Results

3.1. Deficiency of α-galactosidase A

The fibroblasts from all five patients showed a partial deficiency of α-galactosidase and their specific activities ranged between 10% and 30% of the mean of the controls (table 1). Some physicochemical properties of the enzyme from fibroblasts of patients and controls were compared. They had similar pH dependence in the range tested (pH 3.0- 7.0) and only one peak of optimum activity between pH 4.4 and 4.8.

The $K_m$ values of α-galactosidase for the synthetic substrate, as figured from a Lineweaver–Burk plot, in the first four patients were significantly higher than in controls, whereas for the fifth patient this value was similar to that of the controls (table 1).

The $K_m$ values of α-galactosidase from the first four patients was clearly slower than that of the wild-type enzyme for the first 20 min at 51° (fig. 1), whereas the enzyme from the fifth patient, who also showed a lower $K_m$ value, had during the same period a greater thermolability rate than that of the remaining patients. This kind of difference seemed to indicate at first a structural difference between the α-galactosidase of the first four patients and of the controls [21], but the study of the heat inactivation for a longer period, up to 1 hr, showed that the rate of inactivation of the enzyme from controls and from the fifth patient was represented by two lines with different slopes (fig. 1). This suggests the presence in these individuals of a mixture of two enzymes, one being thermolabile (A) and the other thermostable (B), whereas in the majority of the patients (nos. 1-4) only α-galactosidase B is apparent.

3.2 Increase of enzymatic activity with time in culture

The specific activity of α-galactosidase in fibroblasts from patients or controls did not change with time in culture if cells were subcultured at intervals of 3-4 days. However, the specific activity of α-galactosidase increased with time in culture if the same lines derived from a control and a patient were maintained in a stationary phase of growth, that is, in the same petri without subculturing (fig. 2). The specific activity of α-galactosidase from the mutant line increased about 10-fold in a period of 30 days, and that from the control line about 4-fold. The specific activity of β-galactosidase, on the other hand, increased 5-fold in both strains.

No variation of the $K_m$ value and thermolability properties of α-galactosidase concomitant with this increase in activity was observed.
3.3. Deficiency of trihexosylceramide α-galactosidase

The enzyme from various fibroblast lines, partially purified by ethanol precipitation (see Methods) was assayed with the $[^3H]$trihexosylceramide (specific activity 28 μCi/μmole). The α-galactosidase from a control fibroblast line hydrolyzed 1.5 nmoles of $[^3H]$trihexosylceramide/mg protein/hr. The enzyme from patient no. 5 showed one-tenth of the specific activity of the control (0.147 nmoles/mg protein/hr) while no detectable activity was measured in two mutant fibroblast lines (patients nos. 1 and 4). In order to increase the sensitivity of the assay, $[^3H]$trihexosylceramide with high specific activity (240 μCi/μmole, see Methods) was used. It was found that the fibroblast line from patient no. 1 hydrolyzed between 6 and 9 pmoles of this substrate/mg protein/hr, whereas two control lines had an activity of 2.6 and 1.6 nmoles/mg protein/hr, respectively.

4. Discussion

The data presented here constitute biochemical and genetic evidence that α-galactosidase A is deficient in Fabry’s disease. The first four patients showed a residual α-galactosidase activity which amounted to 10–30% of the total activity found in controls, as assayed with the synthetic substrate 4-methylumbelliferyl α-galactoside. This residual α-galactosidase activity was thermostable, like the thermostable component (or α-galactosidase B) found also in the fibroblasts of the controls. The high $K_m$ value found in these four patients was similar to that described for α-galactosidase B [13]. In contrast, the fifth patient, besides the B form of α-galactosidase, also had the A form, as shown by the heat inactivation and by the low $K_m$ value, similar to that of the controls. Even from a clinical point of view, this patient was unusual since he lacked the skin
lesions and had only mild manifestations of his disease [7]. When the activity of trihexosylceramide α-galactosidase was assayed in this patient, about one-tenth of the activity of the controls was found, whereas in patient no. 1 (quite representative of the group of the first four patients) this enzymatic assay showed an activity of about 1/350 of that found in the controls. The demonstration of this low but significant level of activity excludes the possibility that the enzymatic deficiency of Fabry’s disease is caused by a deletion mutation. The occurrence of a milder degree of enzymatic deficiency in the fifth patient is indicative of genetic heterogeneity. Although genetic heterogeneity can be best explained by the presence of different mutations affecting the same structural locus coding for α-galactosidase, other explanations are possible.

It has been proposed, for instance, that the X-linked deficiency of α-galactosidase A in Fabry’s disease is caused by a mutation of an X-linked regulatory locus which, in turn, would cause a deficiency of α-galactosidase A [13]. Although there is no need to postulate the existence of an X-linked regulatory locus, the structural locus for α-galactosidase being X-linked itself [16], the occurrence of regulatory mutations in Fabry’s disease the B form, which is normally sialidase another explanation has been put forward. Since it seems that the A form of this enzyme can be converted into the B form by treatment with sialidase [14], it has proposed that the B form is a precursor of A and that in Fabry’s disease the B form, which is normally sialidated by a sialyl-transferase, is so structurally altered as to become a poor acceptor of sialic residues [14]. This hypothesis would explain the deficiency of α-galactosidase in Fabry’s disease as a result of point mutation of the X-linked locus specifying α-galactosidase.

The presence of sialic acid residues has been shown to be important for the in vivo stability of different sialoglycoproteins [22], and this could justify the presence of two α-galactosidases, one being the native or precursor form and the other final or complete enzyme. However, no definitive conclusion should be drawn until the purification and characterization of both forms of α-galactosidase are completed.

On the basis of the available evidence it is not even possible to decide whether trihexosylceramide is the substrate for only the A form or for both the A and B forms. The data presented here would agree with either alternative.

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References