Sulphamidase activity in leucocytes, cultured skin fibroblasts and amniotic cells: diagnosis of the Sanfilippo A syndrome with the use of a radiolabelled disaccharide substrate

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Summary

1. Sulphamidase activity was assayed by incubation of the radiolabelled disaccharide $O$-$(\alpha$-2-sulphamino-2-deoxy-D-glucopyranosyl)-(1 $\rightarrow$ 3)-L-$\left[6^{-3}\text{H}\right]$idonic acid with homogenates of leucocytes and cultured skin fibroblasts and concentrates of urine derived from normal individuals, patients affected with sulphamidase deficiency disorder [mucopolysaccharidosis type IIIA (MPS IIIA): the Sanfilippo A syndrome], parents of such patients and patients affected with other mucopolysaccharidoses and lysosomal enzyme deficiencies.

2. The assay clearly distinguished affected homozygotes from normal controls, heterozygotes and other mucopolysaccharidoses types.

3. Sulphamidase displayed remarkable thermal stability; reaction rates were constant for at least 24 h at 60°C for leucocyte and 20 h at 37°C for cultured fibroblast preparations. Apparent $K_m$ values for fibroblast sulphamidase were 71 $\mu$mol/l at 37°C and 100 $\mu$mol/l at 50°C; the corresponding $V_{\text{max}}$ values were 21 and 71 pmol min$^{-1}$ mg$^{-1}$ of protein respectively. An incubation temperature of 60°C was used for the routine assay of sulphamidase activity in leucocytes, urine and amniotic cell preparations. The specific activities of fibroblast and amniotic cell sulphamidase, assessed at incubation temperatures of 37°C, were more than 10-fold the leucocyte enzyme activity at 60°C.

4. We recommend the use of radiolabelled disaccharide substrate for the assay of sulphamidase in leucocytes, skin fibroblasts and urine, for the routine enzymic detection of the sulphamidase deficiency disorder of the Sanfilippo A syndrome.

Key words: radiolabelled disaccharide substrate, Sanfilippo A syndrome, sulphamidase deficiency.

Abbreviations: GlcNS-IdoA, $O$-$(\alpha$-2-sulphamino-2-deoxy-D-glucopyranosyl)-(1 $\rightarrow$ 3)-L-$\left[6^{-3}\text{H}\right]$idonic acid; GlcN-IdoA, $O$-$(\alpha$-2-amino-2-deoxy-D-glucopyranosyl)-(1 $\rightarrow$ 3)-L-$\left[6^{-3}\text{H}\right]$idonic acid; MPS IIIA, mucopolysaccharidosis type IIIA.

Introduction

The Sanfilippo A syndrome [mucopolysaccharidosis type IIIA (MPS IIIA)] is caused by a profound deficiency of the lysosomal enzyme sulphamidase [1–3]. This enzyme is involved in the stepwise degradation of the polysaccharide heparan sulphate. A deficiency leads to accumulation of partially degraded heparan sulphate in lysosomes of most tissues which presumably causes the progressive mental retardation in conjunction with mild skeletal deformities of the Sanfilippo syndrome. The syndrome can be diagnosed enzymically by assay of sulphamidase activity in leucocytes or fibroblasts with the commercially available [sulphamino-$\text{35S}$]heparin. There are several disadvantages that arise from the use of heparin as a diagnostic substrate. Most result from the complex polymeric nature of heparin and radiolabelling with short-lived [$\text{35S}$]sulphate. When we assessed a series of
FIG. 1. Degradation of GlcNS-IdoA substrate to GlcN-IdoA product by sulphamidase.

Enzyme preparation

Fibroblasts (sulphamidase-deficient, GM-312-mucopolysaccharidosis type IIIA (MPS IIIA), Sanfilippo A; (α-N-acetylgalactosaminidase)-deficient, GM-156-MPS IIIB, Sanfilippo B; (β-D-glucuronidase)-deficient, GM-121 MPS VII; multiple sulphatase deficient, GM 2407] were obtained from the Human Genetic Cell Repository (Institute for Medical Research, Camden, NJ, U.S.A.). Similar enzyme-deficient cell lines, other mucopolysaccharidosis types (MPS I, II, IVA and VI), mucolipidosis type II and normal fibroblasts were established from skin biopsies taken in or provided to this hospital. MPS IIC fibroblasts (N-acetyl-CoA glcosamine N-acetyltransferase-deficient) were kindly provided by Professor Kurt von Figura (Physiologisch-Chemisches Institut, Westfalische Wilhelms-Universität, Münster, FRG) and Dr M. F. Niermeijer (Erasmus Universiteit, Rotterdam, The Netherlands). Cells from Sanfilippo A patients were deficient in sulphamidase activity when assayed with radiolabelled heparin by a modified method of Whiteman & Young [5]. All lines were maintained in culture as described before and used for the preparation of cell homogenates 6–10 days after subculture [6].

Peripheral blood leucocytes were prepared from 10 ml of heparinized whole blood by sedimentation in aqueous dextran (30 g/l); the final wash was with NaCl solution (150 mmol/l: saline) [7]. Amniotic cells were cultured and harvested as described previously [6]. All cell types were suspended in aqueous Triton X-100 (1 g/l) to a concentration of 5–10 g of protein/l and were disrupted by rapid freezing and thawing six times in solid CO₂/ethanol.

Amniotic fluid and urine supernatants were concentrated 20- and 100-fold respectively in disposable Minicon B15 concentrators (Amicon Corp., Lexington, MA, U.S.A.) at 4°C and washed with 2 vol. of saline.

Enzyme assays

Assay conditions varied for the various preparations of sulphamidase. Incubation mixtures contained approximately 15 μg of protein from cultured fibroblasts, amniotic fluid cell or leucocyte homogenate, or the equivalent of approximately 90 μg of protein from concentrated amniotic fluid supernatants or 10 μg of urine protein in sodium acetate buffer (50 mmol/l), pH 5-0, containing NaH₂PO₄(4 mmol/l) and 680 pmol of GlcNS-IdoA in a final volume of 20 μl. Incubation was at 37°C for 15 h for the

Materials and methods

Substrates

The radiolabelled disaccharide GlcNS-IdoA was prepared by N-sulphation of an unsulphated disaccharide derived from an acid hydrolysate of heparin [4]. The specific radioactivity of the GlcNS-IdoA preparation was 27 Ci/mol. [sulphamino-³⁵S]Heparin (150 mCi/mg) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).
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Substrate

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Distance from origin (cm)

Fig. 2. High-voltage separation of incubation mixtures containing normal fibroblast homogenate (O--O) or Sanfilippo A fibroblast homogenate (●--●).

fibroblast and amniotic cell homogenates and 60°C for 24 h for the leucocyte, amniotic cell supernatant and urine preparations. At the end of the incubation period, products and residual substrate were separated from each other by either ion-exchange chromatography or high-voltage electrophoresis (Fig. 2) as described below. For separation by ion-exchange chromatography the reaction mixtures were applied to small (0.5 cm x 3 cm) glass columns containing approximately 0.4 ml of Dowex I (Cl-form), positioned over scintillation vials. Each column was washed with 2 ml of water to elute the enzyme product into one set of vials and then each column was washed with 2 ml of LiCl (1.0 mol/l) to elute unchanged substrate into a second set of vials. Scintillation fluid (4 ml) was added and the resulting gel counted. The results were corrected for values given by the blank control without protein. As protein did not alter the electrophoretic mobility or ion-exchange binding of the substrate or product, protein-free incubations were used as blanks and run in parallel to the above incubation mixtures. Incubation mixtures not immediately subjected to electrophoresis or ion-exchange chromatography were stored at -20°C. Except where indicated for some cultured fibroblast sulphamidase activities, all substrate/product separations were by high-voltage electrophoresis. Sulphamidase activity was expressed as pmol of GlcN-IdoA produced min⁻¹ mg⁻¹ of protein. It should be noted that the substrate concentration in these incubations is approximately one-half the fibroblast sulphamidase Kₘ. Under these conditions the amount of substrate hydrolysed per minute is far from the maximum that would be observed under saturating conditions. The advantage of using the low substrate concentration is that a relatively high proportion of product is formed.

General methods

High-voltage electrophoresis was performed on Whatman 3MM chromatography paper in formic acid (1.74 mol/l), pH 1.7, at 42 V/cm for 60 min with a Shandon Southern (model L24) HVE system (Shandon Southern Products, Runcorn, Cheshire, U.K.). Radioactivity was measured with a model 6868 ISOCAP/300 Ambient Temperature Liquid-Scintillation Counter (Searle Analytic Inc., Des Plaines, IL, U.S.A.) with conditions as described previously [6]. Protein was determined by the Lowry method [8]. Sulphamidase activity was also assayed with conditions as modified from those described by Whiteman & Young [5] by using [sulphamino-³⁵S]heparin as substrate [4].

Results

The activity of sulphamidase, measured with GlcNS-IdoA as substrate, in a group of individuals at risk for sulphamidase deficiency is shown in Table 1. On the basis of protein content fibroblasts have a considerably higher sulphamidase activity than leucocytes. In fact all leucocyte sulphamidase activity was assayed at
60°C as it was difficult to detect enzyme activity at 37°C. The effect of incubation temperature on fibroblast and leucocyte sulphamidase activity is shown in Fig. 3. Sulphamidase activity is increased in both fibroblasts and leucocyte homogenates with increasing incubation temperatures. Sulphamidase activity in leucocyte homogenates was linear at 60°C for approximately 24 h and at 37°C for at least 20 h in fibroblast homogenates (Fig. 4). The apparent $K_m$ of fibroblast sulphamidase was approximately 71 μmol/l at 37°C and 100 μmol/l at 50°C; the corresponding $V_{max}$ values were 21 and 71 pmol min$^{-1}$ mg$^{-1}$ of protein respectively (Fig. 5). The pH optimum for leucocyte sulphamidase at 60°C was approximately 5-0 compared with 4·5–5·5 for fibroblast sulphamidase at 37°C (Fig. 6). The specific activity of leucocyte or fibroblast sulphamidase determined by either substrate was not independent of protein homogenate concentration in the incubation mixture (Fig. 7). To minimize this effect, routine assays for fibroblast and leucocyte sulphamidase activity were carried

**Fig. 3.** Hydrolysis of GlcNS-IdoA by fibroblast (○—●) or leucocyte (●—●) homogenates as a function of incubation temperature. Standard incubation mixtures containing fibroblast or leucocyte homogenates were held at the indicated temperatures for 15 h and 24 h respectively.
out by using levels of 0.6–1.0 μg of protein/μl of incubation mixture, where the activity was apparently almost independent of protein concentration preparations (Fig. 7).

Sanfilippo A patients had less than 3% of the average sulphamidase activity in both leucocytes and fibroblasts from normal individuals (Table 1). Sulphamidase activity in urine preparations from a normal individual, a Sanfilippo B and a Sanfilippo A patient was 5.80, 6.60 and 0.06 pmol min⁻¹ mg⁻¹ of protein respectively. Sulphamidase activity expressed by fibroblast homogenates from obligate heterozygotes could not be distinguished from the normal control range of enzyme activity (Table 1).

Homogenates of leucocytes and/or fibroblasts
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Sulphamidase derived from the foetus and that from the mother is unknown.

Leucocyte and fibroblast homogenates, assayed at 60°C, from some Sanfilippo A patients had measurable but very low sulphamidase activity. It is not known whether this hydrolytic activity present in these cell preparations represents true residual sulphamidase activity or the action of some other non-specific sulphatase.

Discussion

Ion-exchange chromatography was the most convenient and rapid method for the separation of radiolabelled substrate and product in fibroblast and amniotic cell homogenate incubation mixtures. However, the lower sulphamidase activity present in leucocyte, urine and cell-free amniotic fluid protein preparations required 60°C incubation conditions and the electrophoretic procedure for reproducible separation of the relatively small quantities of radiolabelled product from substrate. Increasing sulphamidase activity with incubation temperature was first described by Whiteman & Young [5] for leucocytes. Results reported in Figs. 3 and 4 highlight the excellent thermal stability of fibroblast and leucocyte sulphamidase. It would appear that increased sulphamidase activity results from thermal activation of the enzyme. An Arrhenius plot of the sulphamidase reaction with fibroblast homogenates suggests that there is a discontinuity in activation energy at about 36°C (Fig. 8). Above this temperature the activation energy is approximately 50.4 kJ/mol, whereas below 36°C the value is approximately 75.6 kJ/mol. However, it should be noted that the substrate concentration used in these experiments is not sufficient to saturate the enzyme. It must be emphasized that these preliminary data are derived from incubations with whole-cell homogenates and it is most important to repeat these studies with purified enzyme preparations.

We conclude that the radiolabelled disaccharide GlcNS-IdoA is a suitable substrate for the routine diagnosis of Sanfilippo A syndrome. With use of this substrate to assess the level of sulphamidase activity in cultured fibroblast, leucocyte and urine preparations, it was possible to distinguish clearly Sanfilippo A patients from other mucopolysaccharidoses patients and normal individuals.

Acknowledgments

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References


