Intermediate forms of human β -N-acetylhexosaminidase lack activity towards 4-methylumbelliferyl β -N-acetylglucosaminide 6-sulphate

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4-Methylumbelliferyl β -N-acetylglucosaminide 6-sulphate was purified from a mixture containing its unsulphated precursor. The substrate was used to test for the presence of functional α -subunits in 'intermediate' forms of human β -N-acetylhexosaminidase in samples of normal and pregnancy serum and in extracts of placenta and lymphocytes from a patient with common acute lymphoblastic leukaemia. Intermediate forms in these samples had no activity towards 4-methylumbelliferyl β -N-acetylglucosaminide 6-sulphate, indicating that they lack α -subunits.

INTRODUCTION

β -N-Acetylhexosaminidase of human tissues and body fluids exists in two main forms, A and B (Robinson & Stirling, 1968). In addition, two or more forms have charge characteristics that cause them to be eluted between A and B on DEAE-cellulose columns and hence are rather loosely referred to as 'intermediate' forms. Although the intermediate forms account for only a small proportion of the total activity in normal tissues and body fluids, they are increased very greatly in maternal serum during pregnancy, when β -N-acetylhexosaminidase P becomes the major form (Stirling, 1971), and in lymphocytes from patients with common acute lymphoblastic leukaemia, which is characterized by an increase in β -N-acetylhexosaminidase I₂ (Ellis *et al.*, 1978). The presence of β -N-acetylhexosaminidase I₂ in leukaemic cells has been used as a marker to define the cALL (common acute lymphoblastic leukaemia) phenotype (Greaves, 1979), and the intermediate forms detected by isoelectric focusing in polyacrylamide gels have been regarded as markers of early haemopoiesis in the lymphoid and myeloid systems and of terminal lymphoid development (Drexler *et al.*, 1986).

The subunit compositions of the A and B forms from human placenta are $\alpha\beta_a\beta_b$ and $2 \times \beta_a\beta_b$ respectively, and the complete amino acid sequences of the prepro- α and β polypeptides have been deduced from the deoxy-nucleotide sequences of cDNA clones (O'Dowd *et al.*, 1985; Myerowitz *et al.*, 1985; Korneluk *et al.*, 1986). Progress in determining the structural relationships between A, B and the intermediate forms has been hampered by their low abundance in normal tissues or in the case of the P form limited supplies of the starting material. Nevertheless, immunological evidence supports the suggestion that β -N-acetylhexosaminidase P is composed entirely of β -subunits (Geiger *et al.*, 1978), and electrophoretic analysis of the I₂ form from human liver

on SDS/polyacrylamide gels showed it to be composed of both α - and β -subunits (Dewji *et al.*, 1986).

Presence of the α -subunit of β -N-acetylhexosaminidase confers an ability to hydrolyse the ganglioside GM₂ and also the synthetic substrates *p*-nitrophenyl and 4-methylumbelliferyl β -N-acetylhexosaminide 6-sulphates (Fuchs *et al.*, 1983; Inui & Wenger, 1984; Ben-Joseph *et al.*, 1985). Hydrolysis of the latter should in principle give a simple and sensitive assay for the presence of α -subunits in intermediate forms of hexosaminidase.

In the present investigation we have assayed intermediate forms of β -N-acetylhexosaminidase in normal and pregnancy serum and in extracts of placenta and lymphocytes from a patient with common acute lymphocytic leukaemia, and we report that in all four cases functional α -subunits are lacking.

EXPERIMENTAL

Materials

4-MUGlcNAc and an impure preparation of 4-MUGlcNAc-6-SO₄ were purchased from Koch-Light Laboratories, Haverhill, Suffolk, U.K. The cation-exchanger DE-52 DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. Dowex 50X-2 (–400 mesh) was supplied by Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were of analytical grade.

Purification of 4-MUGlcNAc-6-SO₄

4-MUGlcNAc-6-SO₄ was purified from an impure preparation of monosulphated MUGlcNAc by adaptation of a method described by Besley & Broadhead (1985). Impure 4-MUGlcNAc-6-SO₄ (70 mg) was dissolved in 2.5 ml of distilled water and loaded on to a DE-52 DEAE-cellulose column (10 ml) equilibrated with distilled water. After being washed with distilled water the column was developed with 60 ml of a linear gradient

Abbreviation used: 4-MUGlcNAc, 4-methylumbelliferyl β -N-acetylglucosaminide.

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of 0–0.5 M-NH₄HCO₃ and then washed with 0.5 M- and 1 M-NH₄HCO₃. The flow rate was 20 ml/h, and 3 ml fractions were collected. The elution profile was monitored by measuring the absorbance at 315 nm, and the fluorescence intensity was measured with the use of excitation at 364 nm and emission at 448 nm on a Locarte fluorimeter. Fractions were examined on Kieselgel 60 t.l.c. sheets (Merck) developed with chloroform/methanol (3:2, v/v) and compounds were detected by spraying with 5% (v/v) H₂SO₄. The fractions containing monosulphated compound (second peak eluted from DE-52 DEAE-cellulose) were pooled, dried by a rotary evaporation at 40 °C, and then resuspended in 9 ml of distilled water and loaded on to a 3.5 ml Dowex 50X-2 column (Na⁺ form) equilibrated with distilled water. The column was washed with distilled water until the absorbance at 315 nm and the fluorescence intensity reached zero, and was then washed with 1 M-NaCl. The sulphated substrate was unretained by the column. The purpose of this step was to convert the sulphated substrate from the ammonium into the sodium salt, but fortuitously free umbelliferone is retarded by the column and consequently the sulphated substrate prepared in this way has a low fluorescence blank. The purity of the final product was tested by t.l.c. and the concentration of sulphated substrate was measured on the basis of the molar absorption coefficient at 315 nm ($\epsilon = 7155 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Assay of β -N-acetylhexosaminidase activity

Enzyme activity was determined with the two fluorogenic substrates 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄. For the first, 20 μ l of sample was mixed with 100 μ l of a solution of 3 mM substrate in 60 mM-citrate/95 mM-phosphate buffer, pH 4.5. After incubation at 37 °C for 5 min, the reaction was stopped by addition of 1 ml of 0.2 M-glycine/NaOH buffer, pH 10.4. For the second, 20 μ l of sample was mixed with 100 μ l of solution of 0.33 mM substrate in 200 mM-citrate/400 mM-phosphate buffer, pH 4.5. After incubation at 37 °C for 60 min, the reaction was stopped as above. For the assay of β -N-acetylhexosaminidase in the placental extract the final concentrations of 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄ were 1.0 mM and 0.55 mM respectively. The fluorescence intensity of the liberated 4-methylumbelliferone was measured (excitation 364 nm, emission 448 nm) on a Locarte fluorimeter (Locarte Co., London W.12, U.K.). One unit of activity is the amount of enzyme that hydrolyses 1 μ mol/min under the conditions described above.

Enzyme preparations

Human placenta was collected immediately after delivery and stored at –20 °C until required. A placental extract was prepared by homogenizing 200 g of tissue in 300 ml of 10 mM-sodium phosphate buffer, pH 6.0. The supernatant recovered after centrifugation at 10000 g for 20 min at 4 °C in a Sorvall RC-5B centrifuge was treated with (NH₄)₂SO₄, and the fraction that was precipitated between 30% and 60% saturation was dialysed against 10 mM-sodium phosphate buffer, pH 6.0. Normal and pregnancy sera were dialysed against 10 mM-sodium phosphate buffer, pH 6.0, before application to the DEAE-cellulose column. A lysate of cALL cells was prepared as described by Orlacchio *et al.* (1986).

Ion-exchange chromatography

A sample of each enzyme preparation (2.5 ml) was loaded on to a column of DE-52 DEAE-cellulose (2 ml) equilibrated with 10 mM-sodium phosphate buffer, pH 6.0. Unretained protein was eluted with the column buffer, and then a linear gradient of NaCl (0–1.0 M in 100 ml of column buffer) was applied. Finally, the column was eluted with 1.0 M-NaCl in the same buffer. Conditions for the elution of β -N-acetylhexosaminidase in the placental extract were identical except that the column volume was 4.5 ml and the gradient was 0–0.5 M-NaCl in 40 ml of column buffer. Protein in the eluates was monitored at 280 nm.

RESULTS AND DISCUSSION

4-MUGlcNAc-6-SO₄ is a useful substrate for the specific detection of β -N-acetylhexosaminidase A, but removal of the unsulphated 4-MUGlcNAc is essential if unambiguous results are to be obtained. The simple method that we describe here for the purification of 4-MUGlcNAc-6-SO₄ allows β -N-acetylhexosaminidases A and B to be distinguished on the basis of their specificities. This is clearly illustrated with respect to

Fig. 1. Separation of placental β -N-acetylhexosaminidases on DEAE-cellulose

An extract of placenta was applied to a DEAE-cellulose column (4.5 ml) equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, and eluted with the same buffer until the unretained protein had been recovered. Protein retained by the column was eluted with a linear gradient of NaCl (—), starting at the point indicated by the arrow, as described in the Experimental section. At the end of the gradient the column was eluted with 0.5 M-NaCl and 1.0 M-NaCl as indicated by the arrows. Fractions (2 ml) were assayed for activity towards 4-MUGlcNAc (●) and 4-MUGlcNAc-6-SO₄ (○).

Fig. 2. Separation of serum β -N-acetylhexosaminidases on DEAE-cellulose

Conditions and other details were those described in the legend to Fig. 1 except that the column volume was 2 ml and 1.2 ml fractions were collected. (a) Normal serum; (b) pregnancy serum.

β -N-acetylhexosaminidases A and B from human placenta in Fig. 1. The origin of this difference in specificity resides in the subunit structures of the two enzymes, the presence of α -subunits conferring the ability to hydrolyse the sulphated substrate (Kytzia & Sandhoff, 1985). β -N-Acetylhexosaminidase B lacks α -subunits and consequently has no detectable activity towards the sulphated substrate. In principle it should be possible to use the sulphated substrate to reveal the presence of α -subunits in minor forms of β -N-acetylhexosaminidase that are not present in sufficient quantities for direct analysis of subunit composition. The intermediate form of β -N-acetylhexosaminidase present in normal serum was reported by Fuchs *et al.* (1983) to hydrolyse *p*-nitrophenyl-GlcNAc-6-SO₄, but we were

Fig. 3. Separation of lymphocyte β -N-acetylhexosaminidases from a patient with common acute lymphoblastic leukaemia on DEAE-cellulose

Conditions and other details were those described in the legend to Fig. 2.

unable to confirm this with 4-MUGlcNAc-6-SO₄ (Fig. 2a). Nor did we find any activity towards 4-MUGlcNAc-6-SO₄ associated with β -N-acetylhexosaminidase P, the predominant form of the enzyme in pregnancy serum (Fig. 2b). This is a matter of practical importance for the identification of pregnant carriers of the Tay-Sachs gene, for whom the standard test based on heat inactivation of serum A gives anomalous results. In their paper on the use of 4-MUGlcNAc-6-SO₄ for the detection of Tay-Sachs heterozygotes, Ben-Yoseph *et al.* (1985) reported that non-carrier pregnant women have serum β -N-acetylhexosaminidase activities towards 4-MUGlcNAc-6-SO₄ in the non-carrier non-pregnant range. It was not evident from these measurements, however, whether β -N-acetylhexosaminidase P was completely lacking activity towards the sulphated substrate. Considering its substrate specificity, its immunological properties (Geiger *et al.*, 1978) and its thermostability it is now apparent that form P is a β -N-acetylhexosaminidase of the 'B type'. The structural relationship between β -N-acetylhexosaminidases B and P is unknown, but the two are clearly distinguished by their clearance rates when infused into the circulation of rats (Bearpark & Stirling, 1977) in a manner suggesting that β -N-acetylhexosaminidase P may be a secretory form of β -N-acetylhexosaminidase B. However, the tissue origins of β -N-acetylhexosaminidase P and the mechanisms leading to its accumulation in maternal blood are still obscure.

β -N-Acetylhexosaminidase I₂, as identified by DEAE-cellulose chromatography, is expressed abnormally in lymphocytes of patients with common acute lymphoblastic leukaemia (Ellis *et al.*, 1978), and a representative elution profile of the enzyme from these cells is given in Fig. 3. The I₂ form, eluted just before the A form in the

salt gradient, showed no activity above blank values towards the sulphated substrate. It would appear that in cALL cells too β -N-acetylhexosaminidase I₂ lacks α -subunits and in that respect resembles the intermediate forms of normal and pregnancy serum. The profile shown in Fig. 1 was chosen because it exhibited a relatively high 'intermediate form activity' for a placental sample, and in this case also there was no activity towards the sulphated substrate. Analysis of the subunit structure of the placental 'intermediate' form (Mahuran *et al.*, 1985) showed it to have a polypeptide of M_r 61000, tentatively identified as an α -subunit precursor, and polypeptides of M_r 35000 and 28000, which were thought to be partially processed β -chains. If this is indeed an α -subunit precursor, it would seem to be in a non-functional form, judged from its lack of activity towards 4-MUGlcNAc-6-SO₄.

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