Human α -L-iduronidase

Catalytic properties and an integrated role in the lysosomal degradation of heparan sulphate

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The kinetic parameters (K_m and k_{cat}) of human liver α -L-iduronidase were determined with a variety of heparin-derived disaccharide and tetrasaccharide substrates. More structurally complex substrates, in which several aspects of the aglycone structure of the natural substrates heparin and heparan sulphate were maintained, were hydrolysed with catalytic efficiencies up to 255 times that observed for the simplest disaccharide substrate to be hydrolysed. The major aglycone structure that influenced both substrate binding and enzyme activity was the presence of a C-6 sulphate ester on the residue adjacent to the iduronic acid residue being hydrolysed. Sulphate ions and a number of substrate and product analogues were potent inhibitors of enzyme activity. Human liver α -L-iduronidase activity towards 4-methylumbelliferyl α -L-iduronide at pH 4.8 had two K_m values of 37 μ M and 1.92 mM with corresponding k_{cat} values of 299 and 650 mol of product formed/min per mol of enzyme respectively, which may explain the wide range of K_m values previously reported for α -L-iduronidase activity toward its substrate. Skin fibroblast α -L-iduronidase activity towards the heparin-derived oligosaccharides was influenced by the same substrate aglycone structural features as was observed for the human liver enzyme. A comparison was made of the effect of substrate aglycone structure upon catalytic activities of the enzymes which act to degrade the highly sulphated regions of heparan sulphate. A model was proposed whereby the substrate is directed from α -L-iduronidase to subsequent enzyme activities to ensure the efficient degradation of heparan sulphate.

INTRODUCTION

α -L-Iduronidase (α -L-iduronide iduronohydrolase, EC 3.2.1.76) is one of several hydrolases involved in the lysosomal degradation of the sulphated glycosaminoglycans heparan sulphate (HS) and dermatan sulphate (DS) (Rodén, 1980; Hopwood, 1989). A deficiency of α -L-iduronidase activity in human results in the tissue accumulation and urine excretion of partially degraded HS and DS fragments, and can lead to the lysosomal storage disorder mucopolysaccharidosis type I (MPS I) (Neufeld & Muenzer, 1989). Severely affected patients may have mental retardation, severe skeletal deformities, coarse facies, corneal clouding and early death, whereas mildly affected patients may have both normal intelligence and life-span with mild skeletal deformities. The considerable variation of clinical presentation of this disorder may be the result of different mutations in the α -L-iduronidase gene (Neufeld & Muenzer, 1989; Hopwood & Morris, 1990). A variety of synthetic iduronides have been evaluated as substrates for α -L-iduronidase activity (Hopwood *et al.*, 1979b; Hopwood & Muller, 1982). Although 4-methyl-

umbelliferyl α -L-iduronide (IdoA-MU) is the most convenient and often used substrate, disaccharide substrates (IdoA-anM, IdoA-anM6S and IdoA-anT4S) chemically derived from heparin or DS have been developed and used as diagnostic substrates (Di Natale *et al.*, 1977; Thompson, 1978; Hopwood & Muller, 1979, 1982; Muller & Hopwood 1984; Hopwood & Morris, 1990). Differences have been observed in the kinetic properties of residual α -L-iduronidase activity toward IdoA-anM6S present in skin fibroblasts from patients at either end of the clinical spectrum; however, intermediate phenotypes are not readily discriminated in this way (Hopwood & Muller, 1979, 1982; Ullrich *et al.*, 1981).

The purification of α -L-iduronidase from human liver and details of some of its catalytic properties toward IdoA-MU and heparin- and DS-derived disaccharide substrates has been reported (Clements *et al.*, 1985a, 1985b, 1989). DNA encoding α -L-iduronidase has been isolated and the gene chromosomally localized at 4p16.3 (Scott *et al.*, 1990, 1991). Other laboratories have reported the purification of human α -L-iduronidase from urine, kidney and lung (Barton & Neufeld, 1971; Rome *et al.*,

Abbreviations used: anM, 2,5-anhydromannitol; anM6S, 2,5-anhydromannitol 6-sulphate; ManNAc, N-acetylmannosamine; GlcNAc, N-acetylglucosamine; GlcNAc6S, N-acetylglucosamine 6-sulphate; GlcNS, 2-sulphaminoglycosamine; IdoA-MU, 4-methylumbelliferyl α -L-iduronide; IdoA-anM, O-(α -L-iduronic acid)-(1 $\rightarrow$ 4)-D-O-2,5-anhydro[1- 3 H]mannitol; IdoA-anM6S, O-(α -L-iduronic acid)-(1 $\rightarrow$ 4)-D-O-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA-anT4S, O-(α -L-iduronic acid)-(1 $\rightarrow$ 3)-D-O-2,5-anhydro[1- 3 H]talitol 4-sulphate; IdoA2S-anM6S, O-(α -L-iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-O-2,5-anhydro[1- 3 H]mannitol 6-sulphate; GlcA2S-anM6S, O-(β -D-glucuronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-O-2,5-anhydro[1- 3 H]mannitol 6-sulphate; GlcNAc6S-IdoA, O-(α -N-acetylglucosamine 6-sulphate)-(1 $\rightarrow$ 3)-L-[6- 3 H]iduronic acid; GlcNAc6S-IdoA2S-anM6S, O-(α -N-acetylglucosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; GlcNS-IdoA2S-anM6S, O-(α -2-sulphaminoglycosamine)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; GlcNS6S-IdoA2S-anM6S, O-(α -2-sulphaminoglycosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA-GlcNS6S-IdoA2S-anM6S, O-(α -2-sulphaminoglycosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA-GlcNH6S-IdoA2S-anM6S, O-(α -L-iduronic acid)-(1 $\rightarrow$ 4)-D-O-(α -glucosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA-GlcNAc6S-IdoA2S-anM6S, O-(α -L-iduronic acid)-(1 $\rightarrow$ 4)-D-O-(α -N-acetylglucosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA2S-GlcNS6S-IdoA2S-anM6S, O-(α -L-iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-O-(α -2-sulphaminoglycosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA2S-GlcNH6S-IdoA2S-anM6S, O-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-O-(α -glucosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; IdoA2S-GlcNAc6S-IdoA2S-anM6S, O-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-D-O-(α -N-acetylglucosamine 6-sulphate)-L-O-(α -iduronic acid 2-sulphate)-O-D-2,5-anhydro[1- 3 H]mannitol 6-sulphate; MPS, mucopolysaccharidosis; HS, heparan sulphate; DS, dermatan sulphate.

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1978; Schuchman *et al.*, 1984a). Enzyme purified from each tissue source has differed in both native protein and subunit molecular mass. Furthermore, different catalytic properties (pH optima, K_m and V_{max} values) have been reported for various preparations of purified enzyme and for α -L-iduronidase in leucocyte and culture human skin fibroblast homogenates. These differences may be due to the tissue source, the method of purification, the presence of modifier proteins or different assay protocols, which have made the comparison of kinetic data between the laboratories difficult. Here we have determined the catalytic properties of purified human liver α -L-iduronidase activity toward heparin-derived substrates using assay conditions which have also been shown to be optimal for iduronate-2-sulphatase (Bielicki *et al.*, 1990), glucosamine-6-sulphatase (Freeman & Hopwood, 1987) and sulphamate sulphohydrolase (Freeman & Hopwood, 1986), which act in sequence with α -L-iduronidase to degrade the highly sulphated regions of HS. This has permitted a comparison of the influence of substrate aglycone structural features on the catalytic activities of the enzymes which are involved in the lysosomal degradation of HS.

MATERIALS AND METHODS

Bovine lung heparin, BSA (crystalline), GlcNAc, GlcNAc6S, ManNAc, anM and GlcNS were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). IdoA-Mu cyclohexylammonium salt was purchased from Calbiochem Corp. (Sydney, Australia).

High-voltage electrophoresis was performed on Whatman 3MM chromatography paper in (a) 0.7 M-formic acid, pH 1.7, at 45 V/cm for 50 min for IdoA-anM6S, (b) 0.7 M-formic acid, pH 1.7, containing 100 mM-NaCl, at 21 V/cm for 150 min for the tetrasaccharide substrates (Freeman & Hopwood, 1992) or (c) 50 mM-sodium acetate buffer, pH 5.1, at 45 V/cm for 50 min for IdoA-anM using a model L-24 high-voltage electrophoresis system (Shandon Southern Products, Runcorn, Cheshire, U.K.). Areas of radioactivity were detected with a Packard model 7201 radiochromatogram scanner (Packard Instruments, Chicago, IL, U.S.A.). Preparative high-voltage electrophoresis was performed as described above in 0.7 M-formic acid, pH 1.7, at 45 V/cm for 60 min. Areas of radioactivity were eluted from the strip with 10% (v/v) ethanol.

Purified human liver α -L-iduronidase (Clements *et al.*, 1989) and iduronate-2-sulphatase (Bielicki *et al.*, 1990) was generously given by Ms. L. Ashton and Ms. J. Bielicki, both from this Department. Iduronate-2-sulphatase activity was determined towards IdoA2S-anM6S (Bielicki *et al.*, 1990). Sulphamate sulphohydrolase was prepared as described by Freeman & Hopwood (1986) and dialysed for 15 h at 4 °C in the presence of BSA (1 mg/ml) against 2 mM-sodium acetate buffer, pH 5.1, containing 30% (v/v) glycerol and 0.1 mM-dithiothreitol. Activity was determined towards GlcNS-IdoA2S-anM6S (Freeman & Hopwood, 1986). An enzyme activity unit corresponds to a substrate turnover of 1 μ mol/min. Protein was determined with BSA as a standard (Lowry *et al.*, 1951).

IdoA2S-anM6S was prepared from the HNO_3 degradation of bovine lung heparin at pH 1.5 by a modification of the method of Hopwood (1979), where the disaccharide fraction, after its isolation by Sephadex G-25 chromatography, was radiolabelled using the method described by Hopwood & Elliott (1981) to a specific radioactivity of 1 Ci/mmol with NaB^3H_4 . IdoA2S-anM6S was isolated after chromatography on a Dowex-1 (Cl^- form) column equilibrated in 50 mM-sodium acetate buffer, pH 5.1, and eluted with a linear gradient of 0–2 M-LiCl in the above buffer (Hopwood, 1979). IdoA-anM6S, IdoA-anM, anM6S and anM were prepared from IdoA2S-anM6S by heating in dilute HCl at 100 °C (Hopwood, 1979).

IdoA2S-GlcNS6S-IdoA2S-anM6S, which was prepared from bovine lung heparin, was de-N-sulphated to produce IdoA2S-GlcNH6S-IdoA2S-anM6S, which was N-acetylated to yield IdoA2S-GlcNAc6S-IdoA2S-anM6S (Bielicki *et al.*, 1990; Freeman & Hopwood, 1991b). The tetrasaccharides IdoA2S-GlcNX6S-IdoA2S-anM6S (X = S, H or Ac) (50 nmol) were quantitatively converted into the corresponding tetrasaccharide IdoA-GlcNX6S-IdoA2S-anM6S by digestion with 5 munits of iduronate-2-sulphatase in 50 mM-sodium acetate buffer, pH 5.4, containing BSA (100 μ g/ml) in a total volume of 200 μ l at 37 °C for 6 h. Products were isolated by preparative high-voltage electrophoresis and stored in 10% (v/v) ethanol at –20 °C. GlcA2S-anM6S, GlcNAc6S-IdOA, GlcNS-IdoA2S-anM6S, GlcNS6S-IdoA2S-anM6S, GlcNAc6S-IdoA2S-anM6S and GlcNH6S-IdoA2S-anM6S were prepared by the methods described by Freeman & Hopwood (1986, 1987, 1989a,b, 1992).

Assay for purified human liver α -L-iduronidase activity

α -L-Iduronidase (25 μ g/ml) was dialysed for 15 h at 4 °C in the presence of BSA (1 mg/ml) against 2 mM-sodium acetate buffer, pH 5.1, containing 100 mM-NaCl, 10% (v/v) glycerol and 0.1 mM-dithiothreitol. The dialysed enzyme was diluted immediately before the assay with the same buffer containing BSA (1 mg/ml). For assays in the absence of albumin, α -L-iduronidase was dialysed against 2 mM-sodium acetate buffer, pH 5.1, containing 500 mM-NaCl, 10% (v/v) glycerol and 0.1 mM-dithiothreitol and diluted immediately before the assay with 2 mM-sodium acetate buffer, pH 5.1, containing 10% (v/v) glycerol and 0.1 mM-dithiothreitol to adjust the final NaCl concentration in the assay to 10 mM.

The pH-activity profiles were determined by the incubation of 10 μ M radiolabelled substrate in 20 mM-sodium formate buffer (pH 2.4–4.5) or 20 mM-sodium acetate buffer (pH 3.3–5.7) with 1 μ l of enzyme solution and any other additives in a total volume of 10 μ l at 37 °C. IdoA-GlcNX6S-IdoA2S-anM6S (X = S and Ac) and IdoA-anM6S were incubated for 25 min with 0.25 ng of α -L-iduronidase protein. IdoA-GlcNH6S-IdoA2S-anM6S and IdoA-anM were incubated for 50 min with 1 ng of α -L-iduronidase protein. All incubations contained a final concentration of 10 mM-NaCl and BSA 100 μ g/ml unless otherwise indicated. Reactions were stopped by freezing in a solid CO_2 /ethanol bath, and products were separated from the substrates by high-voltage electrophoresis. Areas of radioactivity were eluted from the strip in 5 ml of water, 10 ml of scintillation fluid was added, and the radioactivity was determined in a Searle Model 6868 ISOCAP liquid-scintillation counter (Searle Analytic, Des Plaines, IL, U.S.A.). Activity was determined from the percentage conversion of substrate into product and was expressed as μ mol of product formed/min per mg of α -L-iduronidase protein.

α -L-Iduronidase activity toward IdoA-MU (5 nmol) was determined by the incubation of 1 ng of enzyme in 10 mM-sodium maleate (pH 1.4–2.9), 20 mM-sodium formate (pH 2.4–4.5) or 20 mM-sodium acetate (pH 3.3–5.7) in a total volume of 10 μ l for 50 min. The reaction mixture was then transferred to a tube containing 1.7 ml of 200 mM-glycine/NaOH buffer, pH 10.1, and the fluorescence of the product, 4-methylumbelliferone, was determined as described previously (Hopwood *et al.*, 1979b).

K_m and k_{cat} values were determined from Lineweaver-Burk plots at the pH optimum for each substrate in 20 mM-sodium formate buffer, and at pH 4.8 in 20 mM-sodium acetate buffer, using substrate concentrations of 20–500 μ M for IdoA-MU, 1–85 μ M for IdoA-anM and 1–40 μ M for the other substrates. K_m values were also determined at pH 4.8 using substrate concentrations of 0.2–15 mM for IdoA-MU and 0.05–2.3 mM for IdoA-anM6S. All incubations contained NaCl and BSA at final concentrations of 10 mM and 100 μ g/ml.

Assay for human skin fibroblast homogenate and urine supernatant α -L-iduronidase activity

Normal control human skin fibroblast homogenates prepared by the method of Hopwood *et al.* (1979a) and the morning void urine from a normal healthy adult male were dialysed for 16 h at 4 °C against 150 mM-NaCl. Activity towards 10 μ M-IdoA-anM6S was determined in 20 mM-sodium formate buffer (pH 2.4–4.5) and 20 mM-sodium acetate buffer (pH 4.2–5.4) with the addition of 1 μ l of dialysed skin fibroblast homogenate (which contained from 1–4 μ g of protein) or 1 μ l of dialysed urine (0.6 μ g of protein) and incubated at 37 °C for 15 min for fibroblast homogenates and 3 h for urine supernatant. The product was separated from the substrate by high-voltage electrophoresis as described above, and activity was expressed as pmol of product formed/min per mg of protein.

DEAE-Sephacrose chromatography of human skin fibroblast α -L-iduronidase activity

The procedure was a modification of the method of Fujibayashi *et al.* (1984). Normal control skin fibroblasts from three 75 cm³ culture flasks were grown to confluency and harvested as previously described (Hopwood *et al.*, 1979a). A homogenate was prepared by freeze–thawing the fibroblasts in 600 μ l of 0.1 % (v/v) Triton X-100 six times before 2.4 ml of 10 mM-sodium citrate/sodium phosphate buffer, pH 6.0, containing 500 mM-NaCl, was added and the freeze–thaw procedure repeated twice more. The homogenate was centrifuged at 2000 *g* for 20 min. The supernatant was dialysed for 15 h at 4 °C against 10 mM-sodium citrate/sodium phosphate buffer, pH 6.0, containing 10 % (v/v) glycerol and 0.1 mM-dithiothreitol (buffer A) and applied to a 3 ml column of DEAE-Sephacrose equilibrated in the same buffer. α -L-Iduronidase activity was monitored by its activity towards 0.5 mM-IdoA-MU, determined at pH 2.4. The column was washed with 15 ml of buffer A, after which no further α -L-iduronidase activity was eluted. A second form of α -L-iduronidase activity was eluted from the column with 15 ml of buffer A containing 200 mM-KCl (Fig. 5 below). The α -L-iduronidase-containing fractions were pooled, BSA added to give a final concentration of 1 mg/ml and the enzyme dialysed for 15 h at 4 °C against 2 mM-sodium acetate buffer, pH 5.1, containing 100 mM-NaCl, 10 % (v/v) glycerol and 0.1 mM-dithiothreitol. Activity toward 0.5 mM-IdoA-MU and 10 μ M-IdoA-anM6S, at pH 2.4 and 2.7 respectively, was determined by incubation at 37 °C for 1 h. Activity toward 10 μ M-IdoA-GlcNAc6S-IdoA2S-anM6S was determined at pH 3.3, as described above, but with the addition of 5 mM-ManNAc in the incubation mixture to inhibit α -N-acetylglucosaminidase activity. High-voltage electrophoresis was used to separate the products GlcNAc6S-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S from the substrate. Activity toward 10 μ M-IdoA-GlcNS6S-IdoA2S-anM6S was determined at pH 3.6 with the co-incubation of 5 munits of sulphamate sulphohydrolase in the incubation mixture. High-voltage electrophoresis separated the products GlcNS6S-IdoA2S-anM6S, GlcNH6S-IdoA2S-anM6S and GlcNH-IdoA2S-anM6S from the substrate.

RESULTS AND DISCUSSION

Assay of α -L-iduronidase activity

The pH optima for α -L-iduronidase activity towards each of the substrates tested was dependent upon the substrate structure and the presence of NaCl and BSA in the incubation mixture (Fig. 1). Maximal human liver α -L-iduronidase activity toward 44 μ M-IdoA-anM6S, determined in the presence of 125 mM-

NaCl, occurred at pH 3.6, and activity was stimulated 4-fold after the addition of BSA (100 μ g/ml) (Fig. 1a). Activity determined in the presence of BSA at pH 2.7 was stimulated 20-fold when the NaCl concentration was reduced from 125 to 10 mM, and this resulted in an apparent shift in the pH optimum from 3.6 to 2.7 (Fig. 1a). α -L-Iduronidase activity toward 10 μ M-IdoA-anM6S was also maximal at pH 2.7 under similar assay conditions (Fig. 1b). Activity was linear with respect to time for incubations at 37 °C for at least 60 min and for incubations containing from 0.01 to 1 ng of enzyme protein. Increasing the BSA concentration in the incubation mixture up to 1 mg/ml did not further enhance enzyme activity; however, α -L-iduronidase activity assayed in the absence of albumin at pH 3.0 was reduced by 85 %.

The influence of pH upon α -L-iduronidase activity toward heparin-derived disaccharide and tetrasaccharide substrates shown in Fig. 1(b) was determined at substrate concentrations of 10 μ M in the presence of BSA (100 μ g/ml) and 10 mM-NaCl. Maximal activity toward IdoA-anM occurred at pH 3.0; however, IdoA-anM6S was hydrolysed 50 times faster than IdoA-anM at their respective optimal pH. The tetrasaccharides IdoA-GlcNX6S-IdoA2S-anM6S (X = S, Ac and H) were hydrolysed maximally at pH 3.6, 3.3 and 3.0 respectively. Lineweaver–Burk plots demonstrated that the relatively lower activity toward IdoA-GlcNS6S-IdoA2S-anM6S at pH 3.0 compared with that observed at pH 3.6 was not due to substrate inhibition (results not shown). α -L-Iduronidase activity toward both IdoA-GlcNS6S-IdoA2S-anM6S and IdoA-GlcNAc6S-IdoA2S-anM6S was decreased for each substrate by approx. 50 % at pH 2.7, and by 20 % at pH 4.8 when the concentration of BSA was decreased from 100 to 10 μ g/ml. IdoA-GlcNH6S-IdoA2S-anM6S was hydrolysed 3.5-fold and 12-fold slower than the *N*-sulphated and *N*-acetylated tetrasaccharide substrates at their respective pH optima. Weissmann & Chao (1981) had reported that IdoA-GlcNH6S-IdoA2S-anM6S was not hydrolysed by partially purified ox liver α -L-iduronidase. At pH 4.8, the physiological pH for lysosomal activity, α -L-iduronidase activity towards IdoA-anM6S, IdoA-GlcNAc6S-IdoA2S-anM6S and IdoA-GlcNS6S-IdoA2S-anM6S was reduced by 7-fold, 3.2-fold and 1.5-fold compared with activity at the respective pH optima. Activity towards 0.5 mM-IdoA-MU was maximal at pH 2.4 and was 13-fold higher than observed at pH 4.8 (Fig. 1c). Dialysed human urine α -L-iduronidase activity toward IdoA-anM6S was maximal at pH 3.6 when assayed in the absence of albumin (Fig. 1d). However, in the presence of BSA, activity was stimulated by 8-fold at pH 2.4 to shift the apparent pH optima from 3.6 to 3.0.

Human liver α -L-iduronidase activity toward 130 μ M-IdoA-anM6S was reported to have dual pH optima at pH 4.1 and 4.4, whereas activity determined at pH 3.3 was less than 10 % of the maximal activity (Clements *et al.*, 1985b). The enzyme activity was determined in the presence of 125 mM-NaCl, which was necessary for enzyme stability; however, NaCl was reported to be a competitive inhibitor for α -L-iduronidase activity (Hopwood & Muller, 1982; Clements *et al.*, 1985b). We have observed the strong influence of buffer composition and concentration, NaCl and BSA upon the observed enzyme activities for many of the HS-degradative enzymes toward their substrates using enzyme purified from human liver or present in skin fibroblast homogenates. The lysosomal sulphatases in particular are potently inhibited by NaCl, particularly at pH values below 3.9 (Freeman & Hopwood, 1986, 1987, 1989a,b, 1991, 1992; Hopwood, 1989; Bielicki *et al.*, 1990). Incubation in the presence of albumin at low ionic strength apparently maintained the stability of α -L-iduronidase activity, particularly at low pH, without the inhibitory effects of NaCl and resulted in increased activity at low pH compared with the previous study by Clements *et al.* (1985b).

Fig. 1. Hydrolysis of α -L-iduronidase of a variety of substrates as a function of pH

(a) Purified human liver α -L-iduronidase activity toward 44 μ M-IdoA-anM6S in 20 mM-sodium formate buffer (●, ▲, ■) or 20 mM-sodium acetate buffer (○, △) containing 125 mM-NaCl (●, ○), 125 mM-NaCl and BSA (100 μ g/ml) (▲, △) or 10 mM-NaCl and BSA (100 μ g/ml) (■). (b) Purified human liver α -L-iduronidase activity towards 10 μ M-IdoA-anM6S in 10 mM-sodium maleate buffer (○) and 10 μ M-IdoA-anM6S (●), 10 μ M-IdoA-anM (△), 10 μ M-IdoA-GlcNH6S-IdoA2S-anM6S (▲), 10 μ M-IdoA-GlcNS6S-IdoA2S-anM6S (□) and 10 μ M-IdoA-GlcNAc6S-IdoA2S-anM6S (■) in 20 mM-sodium formate or sodium acetate buffer. (No significant difference was obtained for α -L-iduronidase activity in sodium formate or sodium acetate at pH 4.2 or 4.5, and the activities are presented as a single line for clarity.) Each incubation mixture contained 10 mM-NaCl and BSA (100 μ g/ml). (c) Purified human liver α -L-iduronidase activity towards 0.5 mM-IdoA-MU in 10 mM-sodium maleate buffer (●), 20 mM-sodium formate buffer (○) and 20 mM-sodium acetate buffer (■). Each incubation contained 10 mM-NaCl and BSA (100 μ g/ml). (d) Human urine supernatant α -L-iduronidase activity towards 10 μ M-IdoA-anM6S in 20 mM-sodium formate buffer (●), 20 mM-sodium acetate buffer (○), 20 mM-sodium formate buffer containing BSA (100 μ g/ml) (■) and 20 mM-sodium acetate buffer containing BSA (100 μ g/ml) (□). Each incubation mixture contained 10 mM-NaCl.

Kinetic properties

Table 1 shows that, at the respective pH optima, the effect of an aglycone C-6 sulphate ester was for α -L-iduronidase to bind IdoA-anM6S 9 times more strongly than IdoA-anM and to turn it over 15.6 times faster; therefore IdoA-anM6S was hydrolysed 138 times more efficiently. IdoA-MU was a poorly bound substrate, with a K_m value 5.5-fold and 50-fold higher than IdoA-

anM and IdoA-anM6S respectively, but it was turned over 121 and 8 times faster respectively. Comparison of activity towards the disaccharide IdoA-anM6S with activity towards the tetrasaccharide substrates demonstrated the influence of the *N*-substitution and aglycone structures further away from the site of catalysis upon both substrate binding and catalysis. Each of the tetrasaccharides was bound to the enzyme with more than twice the affinity than observed for IdoA-anM6S. Whereas

Fig. 2. Hydrolysis of IdoA-MU by human liver α -L-iduronidase as a function of substrate concentration

For experimental details, see the Materials and methods section. Inset: hydrolysis of IdoA-MU over the concentration range of 2–15 mM.

Table 1. Human liver α -L-iduronidase activity toward a variety of substrates

Substrate	pH	K_m (μ M)	k_{cat} (catalytic-centre activity) (mol/min per mol of enzyme)	$10^{-6} \times$ k_{cat}/K_m (catalytic efficiency)	Relative catalytic efficiency*
IdoA-MU	2.4	110	2132.0	19.4	21.5
	4.8	37	298.7	8.1	9.0
IdoA-anM	3.0	20	17.9	0.9	1.0
	4.8	16	2.0	0.1	0.1
IdoA-anM6S	2.7	2.2	273.6	124.4	138.2
	4.8	10.0	62.7	6.3	7.0
IdoA-GlcNS6S-IdoA2S-anM6S	3.6	1.0	65.0	65.0	72.2
	4.8	2.4	51.0	22.2	24.7
IdoA-GlcNAc6S-IdoA2S-anM6S	3.3	1.0	229.5	229.5	255.0
	4.8	2.9	26.8	9.2	10.2
IdoA-GlcNH6S-IdoA2S-anM6S	3.0	0.9	7.9	8.8	9.8

* Catalytic efficiencies calculated relative to a value for IdoA-anM at pH 3.0 of 1.0.

binding of the substrate to the enzyme was independent of the type of *N*-substitution, the rate of substrate turnover was influenced by the *N*-substitution on the adjacent glucosamine residue. At their respective pH optima, maximal hydrolysis of IdoA-GlcNH6S-IdoA2S-anM6S was 8.2-fold and 29-fold slower than when the substrate processed an aglycone 6-sulphated GlcNS or GlcNAc residue. However, the most catalytically efficient substrate, the tetrasaccharide IdoA-GlcNAc6S-IdoA2S-anM6S, was hydrolysed only 1.9 times more efficiently than the disaccharide IdoA-anM6S.

At pH 4.8, the physiological pH for lysosomal degradation, both IdoA-MU and IdoA-anM were bound 3.0-fold and 1.2-fold more strongly to the enzyme than at their respective pH optima. However, substrate turnover in each case was reduced 8-fold, and the substrates were acted upon 2.4-fold and 7.2-fold less efficiently than at their respective pH optimum. The K_m for activity toward IdoA-anM6S, IdoA-GlcNAc6S-IdoA2S-anM6S and IdoA-GlcNS6S-IdoA2S-anM6S at pH 4.8 was increased from 2.3-fold to 4.5-fold compared with the values obtained at their respective pH optima. However, at pH 4.8, IdoA-anM6S and IdoA-GlcNAc6S-IdoA2S-anM6S were turned over at 23 % and 12 % of the maximum rate of catalysis observed at their respective pH optimum, whereas IdoA-GlcNS6S-IdoA2S-

anM6S was turned over at 80 % of its maximum rate of hydrolysis. Therefore, at pH 4.8, the *N*-sulphated tetrasaccharide was hydrolysed 2.4 times and 3.5 times more efficiently than the *N*-acetylated substrate or IdoA-anM6S respectively, whereas at their pH optima the *N*-sulphated substrate was the poorest substrate.

A major conclusion from the kinetic data summarized in Table 1 is that the substrate structure aglycone to the non-reducing-end iduronic acid residue considerably influences the catalytic efficiency of α -L-iduronidase. The greatest influence of both the binding of the substrate and its turnover by the enzyme resulted from the addition of a C-6 sulphate ester to IdoA-anM to give IdoA-anM6S, which resulted in a 138-fold increase in catalytic efficiency. A similar observation was made for purified human liver iduronate-2-sulphatase activity toward IdoA2S-anM6S, which bound the substrate 4.8 times more strongly and turned it over 13 times faster than for IdoA2S-anM (Bielicki *et al.*, 1990). However, the substrates more complex than IdoA-anM6S, which have increased chain length and penultimate *N*-substituted glucosaminide residues, gave only further 1.8-fold increase in catalytic efficiency for the *N*-acetylated tetrasaccharide, whereas the *N*-sulphated tetrasaccharide was hydrolysed 3.5 times less efficiently than for IdoA-anM6S. Iduronate-2-sulphatase hydro-

Fig. 3. Hydrolysis of 10 μ M-IdoA-anM6S by human liver α -L-iduronidase at pH 2.7 (●) and 4.8 (○) as a function of Na₂SO₄ concentration

For experimental details, see the Materials and methods section.

Fig. 4. Hydrolysis of 10 μ M-IdoA-anM6S by human liver α -L-iduronidase in 20 mM-sodium formate buffer (●) and 20 mM-sodium formate buffer containing 10 μ M-IdoA2S-anM6S (○) as a function of pH

For experimental details, see the Materials and methods section.

lysed its most efficient substrate, IdoA2S-GlcNAc6S-IdoA2S-anM6S, only 3 times more efficiently than IdoA2S-anM6S (Bielicki *et al.*, 1990). The small change in catalytic efficiency was unexpected when compared with results from other heparan sulphate hydrolases. For example, purified sulphamate sulphohydrolase and glucosamine-6-sulphatase each turned over GlcNS6S-IdoA2S-anM6S 3300-fold and 130-fold more efficiently than their most catalytically efficient disaccharide substrates (Hopwood, 1989).

Purified human liver α -L-iduronidase activities toward IdoA-MU, IdoA-anM and IdoA-anM6S were previously determined in 54 mM-sodium dimethylglutarate buffer containing 80–125 mM-NaCl and were reported to have apparent K_m values of 820 μ M, 140 μ M and 116 μ M respectively at pH 4.3 (Clements *et al.*, 1985b). However, when the influence of NaCl concentration upon activity toward IdoA-anM6S and IdoA-MU was determined according to the Debye–Huckel equation (Daniels & Alberty, 1961), Clements *et al.* (1985b) reported that the extrapolation of the linear plot to zero ionic strength afforded theoretical K_m values of 1.8 μ M and 81 μ M for IdoA-anM6S and

IdoA-MU respectively, which are similar to the K_m values observed here (Table 1). α -L-Iduronidase activity from the other tissue sources has been reported to have apparent K_m values ranging from 0.06 to 2.2 mM for activity towards IdoA-MU and from 0.04 to 9.0 mM for activity towards IdoA-anM (Di Natale *et al.*, 1977; Rome *et al.*, 1978; Thompson, 1978; Hopwood *et al.*, 1979a,b; Hopwood & Muller, 1982; Schuchman *et al.*, 1984b; Clements *et al.*, 1985b; Ohshita *et al.*, 1989). When human liver α -L-iduronidase activity toward IdoA-MU at pH 4.8 was determined over a concentration range from 0.02 to 15 mM, Lineweaver–Burk plots revealed the existence of two apparent K_m values of 37 μ M and 1.92 mM with corresponding k_{cat} values of 299 and 650 mol of product/min per mol of enzyme respectively (Fig. 2). Activity toward IdoA-anM6S at pH 4.8 was also observed to have two apparent K_m values of 10 and 475 μ M with corresponding k_{cat} values of 63 and 132 mol of product/min per mol of enzyme respectively. For both substrates, the higher- K_m form acted 23 times less efficiently towards its respective substrate that did lower- K_m form. The observation of two apparent K_m values for human liver α -L-iduronidase activity towards IdoA-MU has not previously been reported, but may offer an explanation for the range of K_m values that have been reported for α -L-iduronidase activity toward IdoA-MU and IdoA-anM. Previous workers have also prepared IdoA-anM which was radiolabelled to a relatively low specific radioactivity compared with that in the present study and therefore may not have detected activity towards low-micromolar concentrations of substrate. It is not known whether the lower- and higher- K_m forms of activity involve a conformational change in the α -L-iduronidase protein. Human skin fibroblast α -L-iduronidase activity towards IdoA-anM6S in both normal controls and mildly affected MPS I patients have been reported to have K_m values of 19 to 53 μ M, whereas severely affected MPS I patient fibroblast activities have elevated K_m values of up to 650 μ M (Hopwood & Muller, 1979, 1982; Ullrich *et al.*, 1981). Patient pathophysiology has been suggested to be related to the level of tissue storage of glycosaminoglycan (Conzelmann & Sandhoff, 1984). If only the higher K_m form of α -L-iduronidase were present in tissues of severely affected MPS I patients, it would necessitate a relatively higher substrate concentration to be present *in vivo* before appreciable substrate degradation would occur. Therefore, the higher levels of stored glycosaminoglycans within the tissues of severely affected patients may be a biochemical determinant of patient phenotype.

Influence of Na₂SO₄ and substrate analogues upon α -L-iduronidase activity

The influence of Na₂SO₄ upon α -L-iduronidase activity was dependent upon the pH of incubation and the substrate structure. Fig. 3 shows the effect of increasing concentrations of Na₂SO₄ upon hydrolysis of IdoA-anM6S. At pH 2.7, activity towards IdoA-anM6S was potentially inhibited by the addition of Na₂SO₄; 50% inhibition of activity occurred with 70 μ M-Na₂SO₄, whereas at pH 4.8, 50% inhibition occurred after the addition of 1 mM-Na₂SO₄. However, activity toward the tighter-binding tetrasaccharide IdoA-GlcNAc6S-IdoA2S-anM6S was inhibited by 200 μ M-Na₂SO₄ by only 17% at pH 3.0, and by 6% at pH 4.8.

α -L-Iduronidase activity toward IdoA-anM6S and IdoA-GlcNAc6S-IdoA2S-anM6S (each at 10 μ M) was inhibited by the addition of oligosaccharides which were substrates for other HS-degrading enzymes, but which α -L-iduronidase itself did not hydrolyse. The inhibition of α -L-iduronidase activity by the substrate analogues, which were present at 10 μ M, was pH-dependent. At pH 2.4 IdoA2S-anM6S inhibited activity towards IdoA-anM6S by 47%, whereas at pH 4.5 activity was inhibited by 11% (Fig. 4). GlcA2S-anM6S inhibited α -L-iduronidase

Fig. 5. DEAE-Sepharose chromatography of skin fibroblast α -L-iduronidase activity

For experimental details, see the Materials and methods section. Elution at arrow 1 was with buffer A and at arrow 2 was with buffer A containing 200 mM-KCl. Fractions indicated were assayed for α -L-iduronidase activity towards IdoA-MU and were pooled as indicated by the braces (Π).

Scheme 1. Proposed pathways for the sequential degradation of IdoA2S-GlcNS6S-IdoA2S-anM6S

The footnote symbols are defined in Table 3.

activity toward IdoA-anM6S at pH 2.7 by 28%. IdoA2S-anM6S inhibited activity towards IdoA-GlcNAc6S-IdoA2S-anM6S by 12% at pH 3.3 and by 8% at pH 4.8. Oligosaccharides possessing

non-reducing terminal glucosamine residues were also potent inhibitors of α -L-iduronidase activity, possibly through competition for the aglycone binding sites required for binding of α -L-iduronidase substrates. GlcNAc6S-IdoA and GlcNAc6S-IdoA2S-anM6S inhibited activity towards IdoA-anM6S at pH 2.7 by 20 and 52% respectively, whereas activity at pH 4.8 was inhibited by 21 and 17% respectively. GlcNS-IdoA2S-anM6S was a potent inhibitor of α -L-iduronidase activity toward both IdoA-anM6S (inhibited by 84% at pH 2.7) and IdoA-GlcNAc6S-IdoA2S-anM6S (inhibited by 75% at pH 3.3 and by 82% at pH 4.8). The monosaccharides, GlcNAc, GlcNAc6S, ManNAc and anM (each at a concentration of 5 mM), had no effect upon α -L-iduronidase activity toward IdoA-anM6S or IdoA-GlcNAc6S-IdoA2S-anM6S at pH 2.7 and 3.3 respectively. Only GlcNAc inhibited activity toward IdoA-anM6S, by 22% at pH 4.8, but had no effect upon α -L-iduronidase activity toward IdoA-GlcNAc6S-IdoA2S-anM6S. anM6S, however, at 10 μ M, inhibited activity toward IdoA-anM6S by 76 and 93% at pH 2.7 and 4.8 respectively, and activity toward IdoA-GlcNAc6S-IdoA2S-anM6S by 55 and 91% at pH 3.3 and 4.8 respectively.

Human liver α -L-iduronidase activity toward IdoA-anM6S at pH 4.3 in the presence of 125 mM-NaCl was previously shown to be potently inhibited by the monosaccharides anM, anM6S, GlcNAc and GlcNAc6S, whereas IdoA2S-anM6S was not an inhibitor (Clements *et al.*, 1985b). However, NaCl, which was a potent inhibitor of iduronate-2-sulphatase activity toward IdoA2S-anM6S (Bielicki *et al.*, 1990), may have inhibited the binding of IdoA2S-anM6S to α -L-iduronidase more potently than it inhibited the binding of IdoA-anM6S. anM6S was also the only monosaccharide tested which inhibited human liver iduronate-2-sulphatase activity toward IdoA2S-anM6S (Bielicki *et al.*, 1990). It is unclear why anM6S is a potent inhibitor of iduronate-2-sulphatase and α -L-iduronidase activity whereas GlcNAc6S has no effect. Thompson (1977) has reported that the DS-derived disaccharide *O*-(α -iduronic acid)-(1 $\rightarrow$ 3)-D-*O*-2,5-anhydrotalitol was readily hydrolysed by liver α -L-iduronidase, whereas the corresponding disaccharide which contained an aglycone *N*-acetylgalactosamine residue was not. Many inhibitors of enzyme activity have a structure similar to a substrate transition state and therefore can bind strongly to the enzyme (Kraut, 1988). The monosaccharide anM6S may approach the transition-state configuration of the aglycone GlcNAc6S residue, which may explain both the potency of inhibition of anM6S and why IdoA-anM6S was unexpectedly hydrolysed with a catalytic efficiency close to that observed for the tetrasaccharide substrates IdoA-GlcNX6S-IdoA2S-anM6S (X = S and Ac).

Skin fibroblast α -L-iduronidase activity towards heparin-derived substrates

The dialysis of normal control skin fibroblast homogenates and assay in conditions of low-ionic-strength buffer resulted in maximal activity toward both 44 μ M- and 10 μ M-IdoA-anM6S at pH 2.7 (which was similar to the human liver enzyme) with an apparent K_m value of 3.8 μ M and a corresponding V_{max} value of 1020 pmol of product/min per mg of homogenate protein. Previously, non-dialysed skin fibroblast activity toward IdoA-anM6S, determined in 50 mM-sodium formate buffer, was maximal at pH 3.6 and had K_m values between 5- and 14-fold higher than observed in this study (Hopwood & Muller, 1979, 1982; Ullrich *et al.*, 1981). Dialysed skin fibroblast β -D-glucuronidase activity toward GlcA-anM6S was optimal at pH 3.0 (Freeman & Hopwood, 1991).

Fujibayashi *et al.* (1984) reported that skin fibroblast α -L-iduronidase existed in two distinct forms which could be separated by DEAE-cellulose chromatography, form A, which

Table 2. Activity of human skin fibroblast from A and B α -L-iduronidase towards a variety of substrates

For experimental details, see the Materials and methods section.

Form	Substrate...	Activity (pmol/min per μ l of enzyme)			
		IdoA-MU	IdoA-anM6S	IdoA-GlcNX6S-IdoA2S-anM6S	
				X = Ac	X = S
A		0.167	0.015	0.035	0.008
B		2.46	0.240	0.483	0.133
	Activity ratio (B/A)...	14.7	16.0	13.8	16.6

degraded HS but not DS, and form B, which degraded DS but not HS. Using a similar protocol, but modified by the addition of salt to the homogenate to ensure complete solubilization of α -L-iduronidase from the membrane components (Clements *et al.*, 1985a) and batch elution of enzyme activity from the DEAE-Sephacrose column (Fig. 5), the two forms of skin fibroblast α -L-iduronidase were examined for activity toward IdoA-MU and the heparin-derived substrates IdoA-anM6S, IdoA-GlcNS6S-IdoA2S-anM6S and IdoA-GlcNAc6S-IdoA2S-anM6S. GlcNS6S-IdoA2S-anM6S, the product of fibroblast α -L-iduronidase activity toward IdoA-GlcNS6S-IdoA2S-anM6S, could be further degraded to GlcNH6S-IdoA2S-anM6S, GlcNS-IdoA2S-anM6S and GlcNH-IdoA2S-anM6S by subsequent sulphamate sulphohydrolase and glucosamine-6-sulphatase activities (see Scheme 1), resulting in the formation of up to four products. Purified human liver sulphamate sulphohydrolase was therefore added to the incubation mixture to degrade the glucosamine-6-sulphatase product GlcNS-IdoA2S-anM6S, since high-voltage electrophoretic separation of the reaction mixture was unable to resolve this product from the substrate. Similarly, IdoA-GlcNAc6S-IdoA2S-anM6S could be sequentially degraded by fibroblast α -L-iduronidase, glucosamine-6-sulphatase, α -N-acetylglucosaminidase, iduronate-2-sulphatase and α -L-iduronidase activities to yield up to five products. The addition of ManNAc (which had no effect upon α -L-iduronidase activity) to the incubation mixture inhibited α -N-acetylglucosaminidase activity, resulting in the formation of two products, GlcNAc6S-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S, which were separated by high-voltage electrophoresis from the substrate. Table 2 shows that both of the forms, A and B, were active toward IdoA-MU and the heparin-derived substrates IdoA-anM6S, IdoA-GlcNS6S-IdoA2S-anM6S and IdoA-GlcNAc6S-IdoA2S-anM6S. The ratio of form B to form A activity towards each of the substrates was similar (from 13.8:1 to 16.6:1), which demonstrates that both forms can degrade heparin-derived substrates. The observed skin fibroblast α -L-iduronidase activity towards each of the substrates tested was influenced by the substrate structure in a manner similar to that observed for the purified liver enzyme, suggesting that the enzymes are kinetically similar. Different forms of purified human liver α -L-iduronidase and iduronate-2-sulphatase have previously been shown to be active towards both HS- and DS-derived disaccharide substrates (Clements *et al.*, 1985b, 1989; Bielicki *et al.*, 1990).

General discussion

The incubation of purified human liver α -L-iduronidase in the presence of BSA and at low ionic strength resulted in the apparent enhancement of α -L-iduronidase activity at lower pH, therefore the shape of the pH-activity profiles for activity toward

the heparin-derived substrates which were substantially different, and the pH optima and K_m values were lower than previously reported. However, these K_m values are consistent with the K_m values observed for the other hydrolytic enzymes which act along with α -L-iduronidase towards heparin-derived substrates.

Optimal conditions for determining α -L-iduronidase activity were similar to those reported for other HS-degradative enzymes purified from human liver (Freeman & Hopwood, 1986, 1987, 1989b, 1992; Bielicki *et al.*, 1990); therefore a comparison can be made of the effect of aglycone structural features upon those enzyme activities from purified human liver which act towards the uronic acid and glucosamine residues in the highly sulphated regions of heparan sulphate. The absolute requirement of the presence of a C-6 carboxy group on the uronic acid residue being attacked has been reported for both α -L-iduronidase and iduronate-2-sulphatase activities (Clements *et al.*, 1985b; Bielicki *et al.*, 1990). Carboxy-group reduction of IdoA-anM6S and IdoA2S-anM6S abolished activity towards either substrate (Clements *et al.*, 1985b; Bielicki *et al.*, 1990); however, the carboxy-reduced substrates were potent inhibitors of activities towards the parent compounds. Disaccharide substrates for both sulphamate sulphohydrolase and glucosamine-6-sulphatase activities where the aglycone C-6 carboxy group was reduced were able to bind strongly to the respective enzyme; however, substrate turnover in each case was poor compared with activity toward the intact substrate (Freeman & Hopwood, 1986, 1987). These results suggest that, for each of the HS-degrading enzymes, the presence of C-6 carboxy groups are not critical for binding of the substrate to each of the enzymes, but are very important for the turnover of substrate. Models have been proposed for both sulphamate sulphohydrolase (Freeman & Hopwood, 1986) and α -L-iduronidase (Clements *et al.*, 1985b) where the charged groups of the enzyme which interact with the carboxy group lie just beyond reach when the substrate first binds, so that stretching of the sulphate ester or the glycosidic bond to be broken results when the charged groups of the enzyme interact with the carboxy group via an induced-fit mechanism.

The presence of an aglycone C-6 sulphate ester on the penultimate residue was important for both of the enzymes acting towards uronic acid residues and gave the greatest single influence upon both the binding of the substrate and the rate of substrate turnover (Bielicki *et al.*, 1990; Clements *et al.*, 1985b; the present paper), whereas the addition of a C-6 sulphate ester to GlcNS-IdoA2S-anM6S increased sulphamate sulphohydrolase activity by 1.6-fold (Hopwood, 1989). The K_m values for the binding of complex tetrasaccharide and trisaccharide substrates to iduronate-2-sulphatase, α -L-iduronidase and glucosamine-6-sulphatase was largely unaffected by the *N*-substituent on the aglycone glucosamine residue (Freeman & Hopwood, 1987; Bielicki *et al.*, 1990). However, the effect of the *N*-substituent upon the rate of substrate turnover was clearly more influential for the glucosamine-6-sulphatase and α -L-iduronidase activities than for iduronate-2-sulphatase. Whereas iduronate-2-sulphatase activity towards the tetrasaccharide IdoA2S-GlcNH6S-IdoA2S-anM6S was 2-fold less efficient than toward either *N*-sulphated or *N*-acetylated substrates (Bielicki *et al.*, 1990), α -L-iduronidase hydrolysed IdoA-GlcNAc6S-IdoA2S-anM6S 3.6-fold and 26-fold more efficiently than it did the *N*-sulphated or non-substituted substrate respectively. Glucosamine-6-sulphatase acted towards the trisaccharide substrate GlcNS6S-IdoA2S-anM6S 10-fold and 300-fold more efficiently than it did towards GlcNAc6S-IdoA2S-anM6S and GlcNH6S-IdoA2S-anM6S respectively (Freeman & Hopwood, 1987). GlcNS6S-IdoA2S-anM6S, therefore, was observed to be the most catalytically efficient substrate for hydrolysis by both sulphamate sulphohydrolase and glucosamine-6-sulphatase. The influence of the

Table 3. Kinetic parameters of enzymes involved in the sequential degradation of IdoA2S-GlcNS6S-IdoA2S-anM6S

Substrate	Enzyme*	K_m (μ M)	$10^{-6} \times$ k_{cat}/K_m (catalytic efficiency)
IdoA2S-GlcNS6S-IdoA2S-anM6S†	IDS	1.4	1568
IdoA-GlcNS6S-IdoA2S-anM6S†	IDUA	1.0	66
GlcNS6S-IdoA2S-anM6S†	G6S	0.25	91
GlcNS-IdoA2S-anM6S†	NS	0.07	665
GlcNH-IdoA2S-anM6S†	NAT§	—	—
GlcNAc-IdoA2S-anM6S†	NAG	—	—
IdoA2S-anM6S†	IDS	4.0	529
IdoA-anM6S†	IDUA	2.2	123
IdoA2S-GlcNS6S-IdoA2S-anM6S‡	IDS	1.4	1568
IdoA-GlcNS6S-IdoA2S-anM6S‡	IDUA	1.0	66
GlcNS6S-IdoA2S-anM6S‡	NS	0.08	942
GlcNH6S-IdoA2S-anM6S‡	G6S	0.35	0.3
GlcNH-IdoA2S-anM6S‡	NAT§	—	—

* Abbreviations: IDS, iduronate-2-sulphatase; IDUA, α -L-iduronidase; G6S, glucosamine-6-sulphatase; NS, sulphamate sulphonyhydrolase; NAT, acetyl-CoA:glucosamine *N*-acetyltransferase; NAG, α -N-acetylglucosaminidase.

† Pathway for initial glucosamine-6-sulphatase activity toward GlcNS6S-IdoA2S-anM6S.

‡ Pathway for initial sulphamate sulphonyhydrolase activity toward GlcNS6S-IdoA2S-anM6S.

§ Skin fibroblast acetyl-CoA:glucosamine *N*-acetyltransferase has been shown to act towards GlcNH-IdoA2S-anM6S, but does not *N*-acetylate GlcNH6S-IdoA2S-anM6S (Freeman & Hopwood, 1992).

|| —, not done.

aglycone IdoA2S residue resulted in the trisaccharide substrate being acted upon by both enzymes over 1000 times more efficiently than from the summation of the individual influences of the aglycone C-6 carboxy and C-2 sulphate ester groups (Hopwood, 1987).

From the data obtained in this and other studies (Freeman & Hopwood, 1986, 1987, 1991b; Hopwood, 1989), the tetrasaccharide substrate IdoA2S-GlcNS6S-IdoA2S-anM6S, which represents the major repeating structure (-IdoA2S-GlcNS6S-) in heparin (Lindahl, 1989), can be used as a model substrate for comparing the activities of the HS-degradative enzymes which act in sequence on the highly sulphated regions of HS (Table 3 and Scheme 1). The kinetic parameters of acetyl-CoA: α -glucosamine-*N*-acetyltransferase and α -*N*-acetylglucosaminidase activity towards more complex heparin-derived substrates have not yet been determined. After iduronate-2-sulphatase and α -L-iduronidase activities there are two possible pathways by which the degradation of GlcNS6S-IdoA2S-anM6S may proceed (Scheme 1). In the first pathway, IdoA2S-GlcNS6S-IdoA2S-anM6S is acted upon sequentially by iduronate-2-sulphatase, α -L-iduronidase, glucosamine-6-sulphatase and sulphamate sulphonyhydrolase such that each enzyme acts towards its particular substrate with greater affinity than the preceding enzyme so the product, once formed, is bound with higher affinity by the next enzyme. After α -*N*-acetylglucosaminidase activity, a similar trend follows for the disaccharide substrates (Table 3). However, sulphamate sulphonyhydrolase acts towards GlcNS6S-IdoA2S-anM6S both with a K_m value 4-fold lower and a catalytic efficiency 10-fold higher compared with glucosamine-6-sulphatase activity towards the same substrate. Human skin fibroblast homogenate activities towards GlcNS6S-IdoA2S-anM6S have shown sulphamate sulphonyhydrolase preferentially de-*N*-sulphates the substrate to produce GlcNH6S-IdoA2S-

anM6S, which is both a poor substrate for subsequent human liver glucosamine-6-sulphatase activity and is not a substrate for *N*-acetyltransferase activity (Freeman & Hopwood, 1987, 1992; Hopwood, 1989). Some mechanism *in vivo* is therefore required to ensure the most efficient pathway for the degradation of GlcNS6S-IdoA2S-anM6S is taken (see below).

Many of the HS-degradative enzymes are potentially inhibited by their own products of hydrolysis and by substrates for other enzymes which they themselves do not act towards (Freeman & Hopwood, 1986, 1987, 1989b, 1992; Hopwood, 1989; Bielicki *et al.*, 1990). Whereas at pH 4.8 both of the glucosamine-6-sulphatase substrates GlcNAc6S-IdoA2S-anM6S and GlcNAc6S-IdoA inhibited α -L-iduronidase activity toward IdoA-anM6S with similar potency, GlcNAc6S-IdoA2S-anM6S inhibited α -L-iduronidase activity more potently at pH 2.7 than did GlcNAc6S-IdoA. Human urine glucosamine-6-sulphatase was observed to bind GlcNAc6S-IdoA and GlcNAc6S-IdoA2S-anM6S with similar affinities at pH 5.0; however, the trisaccharide was bound 8-fold more tightly at pH 3.9 than at the higher pH, whereas skin fibroblast glucosamine-6-sulphatase bound the trisaccharide 7-fold more tightly at pH 3.9 than the disaccharide at pH 4.2 (Freeman & Hopwood, 1992). Both of the substrates apparently also bind to α -L-iduronidase with equal affinity at pH 4.8, but at low pH the trisaccharide binds more strongly to α -L-iduronidase to become a more potent inhibitor than GlcNAc6S-IdoA. The relatively poorer binding of GlcNAc6S-IdoA2S-anM6S at the higher pH value may be due to a decreased influence of the effect of the aglycone IdoA2S group upon binding, since IdoA2S-anM6S was a poorer inhibitor of α -L-iduronidase activity at pH 4.5 than at pH 2.7. At pH 3.9, sulphamate sulphonyhydrolase bound GlcNS-IdoA2S-anM6S with the lowest K_m value observed for the binding of any substrate to an enzyme (Hopwood, 1989). Significantly, GlcNS-IdoA2S-anM6S was the most potent inhibitor of α -L-iduronidase activity. Each of the HS-degradative enzymes which act toward uronic acid or glucosamine residues appear to have similar aglycone substrate-binding sites, which may explain why each of the enzymes studied to date are potentially inhibited by substrates for other HS-degrading enzymes. A high affinity of α -L-iduronidase for the product GlcNS6S-IdoA2S-anM6S, after hydrolysis of IdoA-GlcNS6S-IdoA2S-anM6S, may explain why this tetrasaccharide slowly turned over, since there may be slow dissociation of product from the enzyme, whereas the product GlcNAc6S-IdoA2S-anM6S (from hydrolysis of IdoA-GlcNAc6S-IdoA2S-anM6S) may be less strongly bound to the enzyme. The dissociation of metabolic products from an enzyme may be the slowest step in the overall turnover of substrates, and particularly slow dissociation of product may be obviated by the direct transfer of a metabolite from one enzyme to another via a coupled reaction, for example, substrate channelling. We have previously postulated that HS-degradative enzymes exist in close proximity to each other in a multi-enzyme complex within the lysosome (Hopwood, 1989), similar to that proposed for many mitochondrial degradative and biosynthetic pathways. The close juxtaposition of the active sites of enzymes which act in sequence may allow a channelling or passing of substrates through the system of enzymes rather than relying on prior dissociation of the enzyme-product complex before the next enzyme acts. This would enable the substrate GlcNS6S-IdoA2S-anM6S to be directed from the active site of α -L-iduronidase via glucosamine-6-sulphatase to be acted upon by sulphamate sulphonyhydrolase to enable the most efficient pathway of degradation to proceed.

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