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^{1}H NMR spectroscopic studies establish that heparanase is a retaining glycosidase

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ABSTRACT

Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate side chains of proteoglycans in basement membranes and the extracellular matrix (ECM). Heparanase is implicated in several diverse pathological processes associated with ECM degradation such as metastasis, inflammation and angiogenesis and is thus an important target for anti-cancer and anti-inflammatory drug discovery. Heparanase has been classed as belonging to the clan A glycoside hydrolase family 79 based on sequence analysis, secondary structure predictions and mutagenic analysis, and thus it has been inferred that it is a retaining glycosidase. However, there has been no direct experimental evidence to support this conclusion. Herein we describe ^{1}H NMR spectroscopic studies of the hydrolysis of the pentasaccharide substrate fondaparinux by heparanase, and provide conclusive evidence that heparanase hydrolyses its substrate with retention of configuration and is thus established as a retaining glycosidase. Knowledge of the mechanism of hydrolysis may have implications for future design of inhibitors for this important drug target.
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1. Introduction

Heparanase is an endo-β-glucuronidase that cleaves the heparan sulfate (HS) side chains of proteoglycans found on cell surfaces and as a major constituent of the extracellular matrix (ECM) and basement membranes [1,2]. Apart from roles in a variety of normal physiological processes, heparanase also plays a key role in pathological processes associated with ECM remodeling such as inflammation, angiogenesis and tumor metastasis [3,4], both directly by degradation of the ECM and indirectly by releasing HS-bound growth factors. Heparanase levels are elevated in essentially all human tumors examined to date whilst normal cells usually express little or no heparanase. Studies involving over-expression and silencing of the heparanase gene demonstrate that heparanase plays a direct role in both tumor metastasis and angiogenesis. Moreover, increased heparanase expression correlates with poor post-operative survival of cancer patients [3,4]. Collectively, these studies indicate that heparanase is causally involved in cancer progression and is therefore a valid target for the development of anti-cancer [5–8] and anti-inflammatory [9] drugs. This is supported by numerous in vivo studies showing that treatment with heparanase inhibitors leads to inhibition of tumor growth [10–14]. Several heparanase inhibitors have progressed recently to clinical trials in various cancer indications.

Heparanase is a strict *endo*-glycosidase that hydrolyses the β-glycosidic bonds of glucuronic acid (GlcA) residues at a limited number of internal sites within HS chains to release biologically active fragments of ~5–10 kDa in size. A number of studies have advanced our understanding of the substrate specificity [15] of heparanase and these indicate that heparanase cleaves the linkage between a GlcA unit and an *N*-sulfo glucosamine (GlcN) residue carrying either a 3-*O*-sulfo or a 6-*O*-sulfo group. In addition, heparanase cleaves such linkages with a 2-*O*-sulfated GlcA residue, but not a 2-*O*-sulfated iduronic acid (IdoA) residue, in proximity [16]. This suggests that heparanase recognizes certain sulfation patterns rather than specific monosaccharide sequences [16]. A recent study using structurally defined oligosaccharides indicates that heparanase displays two cleavage modes: consecutive cleavage and gapped cleavage, depending on the structural features at the non-reducing ends of HS [17], thus suggesting a regulatory role in controlling the cleavage site and the extent of degradation of HS.

Glycosidases (glycoside hydrolases) hydrolyze their substrates with either net retention or inversion of configuration at the anomeric centre and are thus categorized as either retaining or inverting glycosidases. Heparanase has been classed as belonging to the clan A glycoside hydrolase family 79 in the carbohydrate-active enzymes database 18] on the basis of sequence analysis, secondary structure predictions and mutagenic analysis [19] and thus a retaining mechanism has been inferred. However, no direct

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experimental evidence has been reported to confirm this conclusion. Sequence analysis and secondary structure predictions infer that heparanase folds with an α/β TIM barrel motif typical of family 79 enzymes. To date, no X-ray crystal structures of heparanase have been published, although a number of homology models have been constructed [20–22]. Given that heparanase is now well established as a promising target for anti-cancer therapeutics, we sought to determine experimentally the mechanism of hydrolysis. Given the differences in the active site architecture of retaining and inverting glycosidases [23], and the lack of an X-ray crystal structure for heparanase, this information could be useful for the design of new heparanase inhibitors.

The stereochemical course of a hydrolysis reaction catalyzed by a glycosidase can often be conveniently determined by the use of ^{1}H NMR spectroscopy [24,25]. In the case of heparanase, monitoring the hydrolysis of its usual substrate, HS or heparin, is complicated by the heterogeneous nature of the substrate and by severe signal overlap in the ^{1}H NMR spectra. In addition, heparanase can cleave the substrate in several locations on the polysaccharide chain resulting in multiple HS/heparin fragments which may themselves be either substrates or inhibitors of the enzyme. The use of a homogeneous, low molecular weight substrate with a single point of cleavage is thus preferred. The pentasaccharide fondaparinux (**1**, AGA*IA$_M$, Scheme 1), was identified as a suitable candidate substrate for this study. Pentasaccharide **1**, a commercially available anticoagulant drug, is a known substrate [26] for heparanase and has been utilized recently to develop an assay [27] suitable for screening inhibitors [10] and studying their kinetics [28]. Pentasaccharide **1** contains one β-GlcA residue and thus a single point of cleavage, and hydrolysis by heparanase results in the formation of only one new reducing sugar (disaccharide **2**).

2. Materials and methods

2.1. Compounds

Fondaparinux (97% purity) was a gift from Dr. Mike West, Alchemia Ltd (Brisbane, Australia).

2.2. Cloning, expression and purification of recombinant human heparanase

Recombinant human heparanase was prepared by previously published procedures [29,30] with some modifications. Insect cell expression was carried out at the University of Queensland Protein Expression Facility in Hi5 cells [31] as these showed better expression and secretion of heparanase compared with Sf9 cells. The optimal time for harvest was found to be 48 h post infection. The cell culture supernatant was concentrated and buffer exchanged (150 mM NaCl, 25 mM Tris–HCl, pH 7.5, 200 mL) using a 30 kDa MW cutoff ultrafiltration cassette (Sartorius) and then loaded onto a heparin-Sepharose column (1 mL, HiTrap HP, GE Healthcare) equilibrated with the same buffer. Unbound protein was removed by washing with buffer before elution with a gradient of 0–1 M NaCl, 25 mM Tris–HCl, pH 7.5 buffer. Fractions containing heparanase were pooled and dialysed with a 12 kDA dialysis membrane (Sigma–Aldrich D9777-100FT) against 150 mM NaCl, 25 mM Tris–HCl, pH 7.0 equilibration buffer at 4 °C for 16 h. Dialysed material was loaded onto a Sepharose SP HiTrap FF (GE Healthcare) column and purified using gradient elution of 0–1 M NaCl, 25 mM Tris–HCl, pH 7.0 buffer. Fractions (2 mL) containing pure heparanase were detected by SDS–PAGE and Western blot and stored at −80 °C. Heparanase concentration (25 μg/mL) was determined using the Bradford assay. Heparanase activity was determined using the previously published WST-1 assay [27].

2.3. NMR spectroscopy

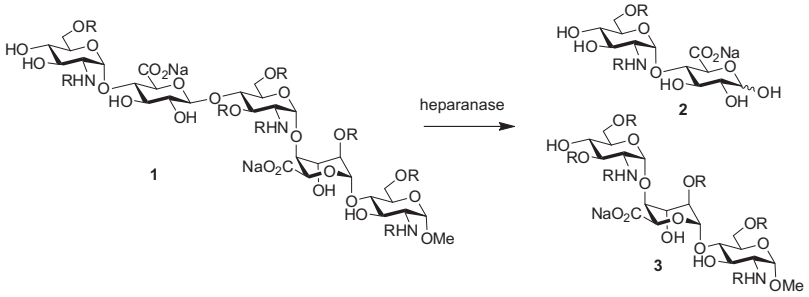
A control spectrum of fondaparinux was recorded containing fondaparinux 100 μL (3.55 mg/mL in 40 mM d_3-sodium acetate buffer). Full assignment of fondaparinux was achieved and ^{1}H and ^{13}C chemical shift data are presented in Table S1. ^{1}H and ^{13}C spectra were recorded at 298 K on a Bruker Avance spectrometer with a TXI cryoprobe operating at 600 and 150 MHz, respectively. Chemical shifts are reported in ppm referenced to internal acetone (^{1}H 2.225 ppm, ^{13}C 31.45 ppm). Spectral assignment was aided by the recording of gradient COSY, DQF-COSY, gradient TOCSY (60 and 120 ms mixing time), ^{13}C attached proton test (APT), ^{1}H^{13}C-HSQC, ^{1}H^{13}C-HSQC-TOCSY (15, 45, 60 and 120 ms mixing times), ^{1}H^{13}C-HMBC and 1D selective COSY, TOCSY (with mixing times ranging from 15 to 180 ms) and NOESY spectra (250 ms). All spectra were acquired using unmodified pulse sequences from the Bruker pulse sequence library.

2.4. Enzymatic analysis of the fondaparinux cleavage

To fondaparinux (100 μL, 3.55 mg/mL in 100 mM d_3-sodium acetate buffer, pH 5.0) and heparanase (100 μL, 80 μg/mL) was added 50 μL of D_2O in a 3 mm NMR tube to give a reaction volume of 250 μL and final buffer concentration of 40 mM. The resulting enzyme reaction was monitored at 600 MHz and 37 °C over a 24 h period. The first spectrum of the heparanase enzymatic cleavage of fondaparinux was recorded after 10 min and subsequent spectra were recorded every 12 min.

3. Results and discussion

Prior to the enzymatic study, all proton chemical shifts for **1** were fully assigned by a combination of 1D and 2D NMR spectroscopic techniques, aided by reference to Torri et al. [32]. Additionally, ^{13}C assignments were made for **1** using a combination of APT, HSQC, and HSQC-TOCSY experiments. All spectra were calibrated



Scheme 1. Hydrolysis of fondaparinux **1** (AGA*IA$_M$) by heparanase to give products **2** and **3**. R = SO_3Na. Abbreviations: A = GlcNS,6S; G = GlcA; A* = GlcNS,3S,6S; I = IdoA2S.

relative to internal acetone, $^{1}H = 2.225$ ppm, $^{13}C = 31.45$ ppm. Complete ^{1}H and ^{13}C NMR assignments are presented in Table S1 (see Supplementary Data).

In the heparanase catalyzed reaction of **1** the glycosidic bond between the (1 → 4)-α-GlcA (G) and the 2-*N*-sulfo-3,6-di-*O*-sulfo-(1 → 4)-α-D-GlcN (A_s^*) is hydrolysed. It has not been firmly established if this reaction proceeds with retention of the β-anomeric configuration of the GlcA or inversion to the α-anomer. To investigate whether this reaction proceeds with retention or inversion of anomeric configuration, the progress of the enzymatic reaction was monitored using ^{1}H NMR spectroscopy by close examination of the anomeric region of the spectrum of **1** as the anomeric proton signals of each of the sugar residues provide a convenient window into the progress of the enzymatic reaction.

An initial control spectrum of **1** (100 μL, 3.55 mg/mL in 40 mM d_3-sodium acetate buffer, pH 5.0) was recorded without the presence of heparanase. For the enzymatic reaction, to a solution of **1** (100 μL, 3.55 mg/mL in 100 mM d_3-sodium acetate buffer, pH 5.0) and heparanase (100 μL, 25 μg/mL) was added D_2O (50 μL) in a 3 mm NMR tube to give a reaction volume of 250 μL and a final buffer concentration of 40 mM. The resulting enzymatic reaction was monitored at 600 MHz and 37 °C over a 24 h period. The first spectrum of the heparanase enzymatic cleavage of **1** was recorded after 10 min and subsequent spectra were recorded every 12 min (Fig. 1). Given that the cleavage occurs between the (1 → 4)-β-GlcA (G) and the 2-*N*-sulfo-3,6-di-*O*-sulfo-(1 → 4)-α-D-GlcN (A_s^*) it was of interest to carefully analyse the anomeric signals of the GlcA (G). After 10 min, most notable was the appearance of an additional β-anomeric signal at 4.66 ppm due to β-GlcA of the cleavage product 2*N*-sulfo-6-*O*-sulfo-α-D-GlcN-(1 → 4)-β-GlcA (**2**), firmly establishing that the reaction proceeds with overall retention of anomeric configuration and that heparanase is thus a retaining glycosidase. Additionally after approximately 45 min there appeared an entirely new anomeric signal at 5.23 ppm that increased in intensity as the reaction proceeded. This signal is due to mutatrotation of the newly formed reducing end of the disaccharide **2** to the α-anomer of GlcA.

The final equilibrium anomeric ratio of the reaction could be approximated by examination of the anomeric signals of *N*-sulfo-6-*O*-sulfo-α-D-GlcN (A_s^{nr}). Before hydrolysis of **1** the anomeric signal of *N*-sulfo-6-*O*-sulfo-α-D-GlcN (A_s^{nr}) resonated at 5.62 ppm. This signal decreased as the reaction proceeded and initially a signal appeared at 5.65 ppm which grew in intensity and is due to the anomeric signal of the (A_s^{nr}) of the disaccharide **2** [or (A_s^{nr})-α-(1 → 4)-Gβ], however, an additional peak became evident at 5.63 ppm which is due to the anomeric signal of the (A_s^{nr}) of disaccharide **2** [or (A_s^{nr})-α-(1 → 4)-Gβ]. Comparison of the integration of these anomeric signals, (A_s^{nr})-α-(1 → 4)-Gβ: (A_s^{nr})-α-(1 → 4)-Gα gave a final anomeric ratio of 59:41 on complete hydrolysis of **1**.

The heparanase catalyzed hydrolysis of **1** was slow, even at 37 °C, and took 24 h for completion. This reflects the fact that the 3-*O*-sulfo group of A_s^* renders **1** a less than ideal substrate compared with its non-3-*O*-sulfated congener ($AGAIA_M$) [26]. At 25 °C the reaction was so slow as to be undetectable by NMR spectroscopy and the possibility of performing saturation transfer difference (STD) NMR spectroscopic studies at lower temperatures was considered. The current absence of an X-ray crystal structure of heparanase mean that such studies could potentially provide useful information about which functional groups on pentasaccharide **1** interact with residues in the active site of heparanase, provided there was no turnover of substrate. Unfortunately, the increased concentrations of heparanase required for the STD NMR experiments resulted in cleavage of substrate, even at 5 °C.

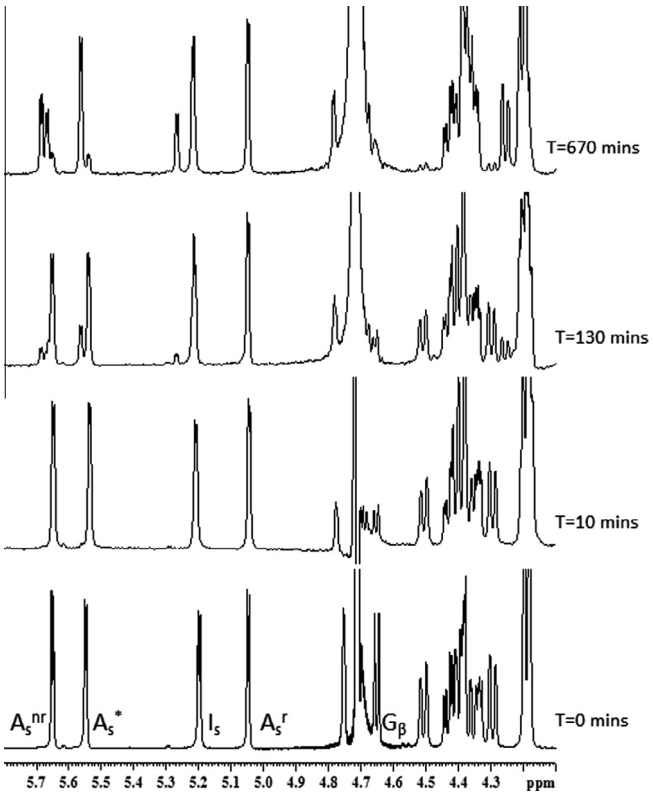


Fig. 1. Overlayed ^{1}H NMR spectra of the hydrolysis of pentasaccharide **1** catalysed by heparanase at different time points.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.079.

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