The Heparin/Heparan Sulfate 2-O-Sulfatase from Flavobacterium heparinum

MOLECULAR CLONING, RECOMBINANT EXPRESS,ATION, AND BIOCHEMICAL CHARACTERIZATION*

James R. Myette‡§, Zachary Shriver‡§, Chandra Claycamp†, Maitland W. McLean**, Ganesh Venkataraman‡‡, and Ram Sasisekharan‡‡‡

From the *Division of Biological Engineering, ‡Department of Biology, and ††Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and **Grampian Enzymes, Ltd., Nisthouse Harrow, Orkney KW17 2LQ, United Kingdom

Received for publication, November 8, 2002, and in revised form, December 16, 2002
Published, JBC Papers in Press, January 7, 2003, DOI 10.1074/jbc.M211420200

Heparan sulfate glycosaminoglycans are structurally complex polysaccharides critically engaged in a wide range of cell and tissue functions. Any structure-based approach to study their respective biological functions is facilitated by the use of select heparan sulfate glycosaminoglycan-degrading enzymes with unique substrate specificities. We recently reported of one such enzyme, the Δ4,5-glycuronidase cloned from Flavobacterium heparinum and recombinantly expressed in Escherichia coli (Myette, J. R., Shriver, Z., Kiziltepe, T., McLean, M. W., Venkataraman, G., and Sasisekharan, R. (2002) Biochemistry 41, 7424–7434). In this study, we likewise report the molecular cloning of the 2-O-sulfatase from the same bacterium and its recombinant expression as a soluble, highly active enzyme. At the protein level, the flavobacterial 2-O-sulfatase possesses considerable sequence homology to other members of a large sulfatase family, especially within its amino terminus, where the highly conserved sulfatase domain is located. Within this domain, we have identified by sequence homology the critical active site cysteine predicted to be chemically modified as a formylglycine in vivo. We also present a characterization of the biochemical properties of the enzyme as it relates to optimal in vitro reaction conditions and a kinetic description of its substrate specificity. In particular, we demonstrate that in addition to the fact that the enzyme exclusively hydrolyzes the sulfatase at the 2-O-position of the uronic acid, it also exhibits a kinetic preference for highly sulfated glucosamines within each disaccharide unit, especially those possessing a 6-O-sulfate. The sulfatase also displays a clear kinetic preference for disaccharides with β1−4 linkages but is able, nevertheless, to hydrolyze unsaturated, 2-O-sulfated chondroitin disaccharides. Finally, we describe the substrate-product relationship of the 2-O-sulfatase to the Δ4,5-glycuronidase and the analytical value of using both of these enzymes in tandem for elucidating heparin/heparan sulfate composition.

* This work was supported by National Institutes of Health (NIH) Grants GM 57073 and CA90940. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†† Recipient of NIH/NIH Training Grant 5T32GM08334.
§§ To whom correspondence should be addressed: Division of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-258-9494; Fax: 617-258-9409.

Sulfated glycosaminoglycans such as heparin and the related heparan sulfate (HSGAGs) are complex, linear carbohydrates possessing considerable chemical heterogeneity. Their structural diversity is largely a consequence of the variable number and position of sulfates present within a single HSGAG chain. Because of their highly anionic character, these polysaccharides historically have been relegated to an exclusively structural role, namely as a sort of hydration gel and scaffold comprising the extracellular matrix. Contrary to this limited perception, however, HSGAGs actually play an important and dynamic function in many critical biological processes ranging from development to tissue repair to apoptosis. These polysaccharides are also central players in several pathological conditions such as cancer, angioinvasion, neurodegenerative diseases such as Alzheimer’s disease, arthritis, and microbial infectivity. HSGAGs do so as part of proteoglycans found at the cell surface and within the extracellular matrix, where they mediate signaling pathways and cell-cell communication bymodulating the bioavailability and temporal-spatial distribution of growth factors, cytokines, and morphogens. We must now therefore appreciate the fact that HSGAG structure and function are inextricably related.

A study of the HSGAG structure-function paradigm requires the ability to determine both the overall composition of biologically relevant HSGAGs as well as ultimately ascertaining their actual linear sequence. Invaluable toward this realization has been the availability of several chemical and enzymatic reagents that are able to cleave HSGAGs in a structure-specific fashion. One example of an important class of GAG-degrading enzyme is the heparinases (heparinases) originally isolated from the Gram-negative soil bacterium Flavobacterium heparinum. Each of the three heparinas encoded by this microorganism cleaves both heparin and heparan sulfate with a substrate specificity that is generally based on the differential sulfation pattern that exists within each GAG chain. In fact, F. heparinum uses several additional enzymes in an apparently sequential man-

1 | The abbreviations used are: HSGAG, heparin/heparan sulfate glycosaminoglycan; U, uronic acid with a Δ4,5 double bond; 2-O ΔN−15; recombinant 2-O-sulfatase lacking NH2-terminal signal sequence composed of first 24 amino acids; MALDI, matrix-assisted laser desorption ionization; GAG, glycosaminoglycan; RP-HPLC, reverse phase high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; ADA, [(carbamoylmethyl)imino]diacetic acid.
2 | Whereas Flavobacterium is the commonly used nomenclature, the genus Pedobacter is the currently accepted taxon for this heparinolytic bacteria.
Cloning and Expression of the Flavobacterial 2-O-Sulfatase

Reagents—Heparin and chondroitin disaccharides were purchased from Calbiochem. Unfractionated heparin was obtained from Celsus Laboratories (Cincinnati, OH). Materials for AZAP II genomic library construction, screening, and phagemid excision, including bacteriotherapy agent host strain XL1Blue MRF and the helper-resistant strain SOLR, were obtained from Stratagene (La Jolla, CA) and used according to the manufacturer’s instructions. Restriction endonucleases and PCR enzymes were purchased from New England Biolabs (Beverly, MA). DNA oligonucleotide primers were synthesized by an Invitrogen custom primer service (Carlsbad, CA). TOP10 chemically competent cells for oligonucleotide primers were synthesized by an Invitrogen custom primer service (Carlsbad, CA). TOP10 chemically competent cells for PCR cloning and subcloning were also obtained from Invitrogen.

Purification of the Flavobacterium Heparinum 2-O-Sulfatase and Subsequent Proteolysis—The 2-O-sulfatase was purified from 20-liter fermentation cultures essentially as described (22). Briefly, the large scale cultures were grown at 25 °C for 48 h. Cell lysates were obtained by a repeated passage of a resuspended cell pellet through an Amino French pressure cell. The homogenate was clarified by centrifugation (37,000 × g). The 2-O-sulfatase was purified from this cell-free supernatant by employing five chromatographic steps carried out in the following sequence: cation-exchange (CM-Sepharose CL-6B) → hydroxyapatite (Bio-Gel HTP) → gel filtration (Sephadex G-50) → taurine-Sepharose CL-4B → blue Sepharose CL-6B. 2-O-Sulfatase activity was measured at each chromatography step as described (22). Fractions from initial CM-Sepharose chromatography were also assayed for heparinase, chondroitinase (AC and B), and 4,5-glycuronidase activities as well as any co-eluting 6-O- or N-sulfatase activities. The highly purified 2-O-sulfatase pool from the final blue Sepharose chromatography step was free from any contaminating glycosaminoglycan-degrading activity.

Generation of 2-O-Sulfatase Peptides and Protein Sequencing—In preparation for proteolysis, the purified flavobacterial sulfatase was first desalted by reverse phase chromatography (RP-HPLC) on a 150 × 4.6-mm C4 column (Phenomenex, Torrance, CA). Protein was eluted by applying a linear gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid. During this elution, both a major and minor protein peak were detected by UV absorbance (Fig. 1A). The two separate fractions were lyophilized to dryness and resuspended in 50 μl of denaturation buffer (8 m urea, 0.4 M ammonium bicarbonate, pH 7.5). Both protein fractions were digested with modified trypsin for ~18 h at 37 °C. Tryptsin was added at a 1:40 ratio (w/w) relative to each sulfatase fraction. Prior to proteolysis, cysteines were first subjected to reductive cleavage by the addition of 5 μl dithiothreitol for 6 h at 50 °C, followed by the addition of 20 μl iodoacetic acid for 30 min (room temperature). The alkylation reaction was quenched by the addition of 50 μl of denaturation buffer. The resulting peptides were resolved by RP-HPLC on a 250 × 2-mm C4 column using a linear gradient of 2–80% acetonitrile in 0.1% trifluoroacetic acid carried out over a 120-min time course. Select peptides corresponding to chromatography peaks 2, 3, 4, 5, and 8 (Fig. 1B) were sequenced using an on-line model 120 phenylthiohydantoin-derivative analyzer (Biopolymer Laboratories, Massachusetts Institute of Technology).

Molecular Cloning of the Flavobacterial 2-O-Sulfatase—The 2-O-sulfatase was cloned from a ZAP II flavobacterial genomic library essentially as described (21). A 600-base pair DNA plaque hybridization probe was generated by PCR using degenerate primers 5′-ATGAGAATHCC-NACNATH-3′ (forward) and 5′-DATNGTTCATCCNRTGTYG-3′ (reverse). PCR was carried out for 35 cycles using a 52 °C annealing temperature and 2-min extensions at 72 °C. The specificity of this probe was established by DNA sequence analysis, which indicated a direct correspondence of its translated sequence to peak 1 tryptic peptides. Based on this information, the nondegenerate primers 5′-CATACCG-TATGGCGGATTAT-3′ (forward) and 5′-GATGTTTGGGATGATG-3′ (reverse) were subsequently used in place of the original degenerate primers. PCR-amplified DNA probe was gel-purified and subsequently 32P-radiolabeled using the Prime-it II random priming kit (Stratagene). Plaques were lifted on to nylon membranes (Nytran Supercharge; Schleicher and Schuell), and DNA was cross-linked to each filter by UV irradiation. Hybridization probes were completed overnight at 42 °C according to standard methods and solutions (23). Positive clones were visualized by phosphorimaging (Amersham Biosciences) and/or autoradiography. Clones were further purified by secondary and tertiary screens, and the recombinant phage was excised as a double-stranded phagemid (pBluescript) as described by the manufacturer (Stratagene). Recombinants were confirmed by DNA sequencing using both T7 and T3 primers. Insert size was determined by restriction mapping of pBluescript inserts using NdeI, XhoI, and XhoI. The full-length sulfatase gene (phagemid clone SHA) was subcloned into the T7-based expression plasmid pET28a in three steps. In the first PCR step, NdeI and XhoI restriction sites were introduced at the 5′ and 3′ termini of the 2-O-sulfatase coding sequence by using primers 5′-TGTTCAGATCATGAAGATGTAACATATCGAACGAAAG-3′ and 5′-GTTCT-CCAGATCTTATTTTITATATGCTAATAACGGATCC-3′, respectively.

At the same time, the NdeI restriction site already present within the sulfatase gene was starting at position 1050 (Fig. 2) was abolished by silent mutagenesis (CATATG → CATCTG) using the mutagenic primers 5′-GATATTATCCCACTATCCTGTTCCGTTGCGGAAAG-3′ and 5′-TTCGGGCAAAGGCACAGATGGTGGGGGATAATATC-3′, with the A to T transversion underlined. In the second step, the final PCR product was purified and ligated into the TOPO TA PCR cloning vector TOPO TA 2.1 (Invitrogen) following the addition of 3′-dA overhangs with 0.5 units of T4 polymerase and 300 μM dATP (10 min, 72 °C). Ligated DNA was transformed into One-shot TOP10 chemically competent cells. Positive clones were identified by blue/white colony selection and confirmed by PCR colony screening. In the third step, the 1.5-kb sulfatase gene was excised from PCR 2.1 TOPO and passed into pET28a (Novagen) as an NdeI-XhoI cassette. Final expression clones were confirmed by plasmid DNA sequencing.

A 2-O-sulfatase amino-terminal truncation lacking the first 24 amino acids (2-D Δ24) was PCR-cloned as above, except the forward primer 5′-TCTAGACATATCGAACCTCAAAGATGGAGATGACGCT-3′ was used in addition to the NdeI site in the original forward primer listed. In the second PCR step, the 2-O-sulfatase-specific sequence begins with Gln25 (Fig. 2) and reads according to standard methods and solutions (23). Posi-
to 30 ml of diluted enzyme over the course of several hours while gently mixing by inversion at 4 °C. Substantial precipitation of the sulfatase routinely occurred during the cleavage reaction. Thrombin was recovered by the addition of streptavidin-agarose using the thrombin cleavage capture kit (Novagen). Capture was carried out at 4 °C for 2 h with gentle mixing. Bound thrombin was collected by centrifugation for 5 min at 500 × g. Supernatant containing soluble 2-O-sulfatase was then dialyzed at 4 °C against 12 liters of enzyme dilution buffer using 20.4-mm diameter Spectra/Por dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA) with a 10,000 molecular weight cut-off. Following dialysis, the purified sulfatase was concentrated using a Centricon YM10 ultratitution device (Millipore Corp.). Enzyme was stable for at least 2 weeks at 4 °C. Long term storage was carried out at −85 °C in the presence of 10% glycerol without any substantial loss of activity due to freezing and thawing.

Protein concentrations were determined by the Bio-Rad protein assay and confirmed by UV spectroscopy using a theoretical molar extinction coefficient (ε280) of 77,380 M⁻¹ cm⁻¹ for 2-O-ΔN⁴⁴ with the histidine tag removed. Protein purity was assessed by silver staining of SDS-polyacrylamide gels.

**Molecular Mass Determinations by MALDI Mass Spectrometry**—The molecular weight of the 2-O-sulfatase NH₂-truncated enzyme (2-O ΔN⁴⁴) was determined by MALDI mass spectrometry essentially as described (19). The NH₂-terminal histidine tag of the recombinant protein was cleaved by thrombin prior to mass analysis. 1 μl of a 2-O-sulfatase solution (diluted in water to 0.5 mg/ml) was added to 1 μl of a saturated sinapinic acid matrix solution previously deposited on to the plate. The observed mass of the recombinant enzyme was corrected according to an external calibration using mass standards.

**2-O-Sulfatase Assay and Determination of Biochemical Reaction Conditions**—2-O-sulfatase activity was measured using the unsaturated disaccharide substrate ΔU₂NS in a 10% glycerol solution lacking a sulfate at the 2-OH-position. Standard reactions included 50 mM imidazole, pH 6.5, 50 mM NaCl, 500 μM disaccharide, and 25 mM enzyme (2-O ΔN⁴⁴) in a 20-μl reaction volume. The reaction was carried out for 30 to 60 min. Prior to its addition, the enzyme was serially diluted to 250 nM in ice-cold 10 mM imidazole buffer. The assay was initiated by the addition of 2 μl of this 10% enzyme stock to 18 μl of reaction mixture. The enzyme was inactivated by heating at 95 °C for 5 min in preheated 0.5-ml Eppendorf tubes. Desalting at the 2-OH-position of the disaccharide was measured by capillary electrophoresis. Resolution of substrate and product was achieved under standard conditions described for HSGAG compositional analyses (19). Activity was generally measured as mol of desulfated product formed and was calculated from the measured area of the product peak based on molar extinction coefficients at 232 nm.

**Substrate Specificity and Kinetics**—For substrate specificity experiments, the following heparin disaccharide substrates were used: ΔU₂NS, ΔU₄SH₅GlcNAc, and ΔU₂SH₅GlcNAc. In addition, the chondroitin disaccharides ΔU₂GalNAc, and ΔU₂SH₅GalNAc were also studied. Di- and trisulfated disaccharide substrate concentrations for each respective substrate were varied from 0.1 to 4 mM. Initial rates (V₀) were extrapolated from linear activities representing <20% substrate turnover and fit to pseudo-first-order kinetics.

**RESULTS**

**Molecular Cloning and Recombinant Expression of the F. heparinum 2-O-Sulfatase**—As a first step toward the cloning of the 2-O-sulfatase gene, we purified the enzyme directly from the native bacterium followed by a partial determination of its amino acid sequence. Enzyme purification was essentially as previously described (22). After a five-step chromatographic fractionation of flavobacterial lysates, we achieved a greater than 3000-fold purification of sulfatase activity. Further fractionation of this activity by reverse phase HPLC chromatography yielded two separate polypeptides (Fig. 1A). Both proteins were subjected to trypsin digestion, and the resultant peptides were likewise purified by reverse phase HPLC (Fig. 1B). From sequential digestion mixture, we identified a conserved sulfatase domain as annotated in the Sequence analysis of this amplified DNA indicated a translated product of 468 amino acids in length (464 amino acids from the first methionine) and whose primary sequence included all of the sulfatase peptides for which we had obtained sequence information (Fig. 2). Based on its amino acid composition, the encoded protein is quite basic (theoretical pI of 8.75), with 67 basic side chains comprising ~14% on a molar basis. The putative sulfatase also possesses 8 cysteines in addition to 46 aromatic amino acids.

Upon a closer examination of its primary sequence, we also identified a conserved sulfatase domain as annotated in the
Cloning and Expression of the Flavobacterial 2-O-Sulfatase

Protein Family Database. This signature domain included the consensus sequence (C/S)XPXRXXXX(S/T)G (25), comprising, at least in part, the sulfatase active site and possessing the cysteine (underlined) that is presumably modified as a formylglycine in vivo (26). The putative 2-O-sulfatase that we cloned from F. heparinum exhibits substantial homology to many members of a highly conserved sulfatase family (data not shown) (27, 28). A structurally oriented description of this homology and its correlation to enzyme function is found in the accompanying paper (40).

From this sequence information, we were confident that we had indeed cloned a sulfatase from the flavobacterial genome. To ultimately establish its functionality, we next set out to recombinantly express this protein in E. coli. The full-length gene (beginning at the first methionine noted in Fig. 2) was subcloned into the T7-based expression vector, pET28a, for expression as an NH$_2$-terminal 6× histidine-tagged protein to facilitate purification. Induction with isopropyl-1-thio-β-D-galactopyranoside led to a limited soluble expression of a polypeptide whose apparent molecular weight roughly corresponded to the theoretical mass of the fusion protein (54 kDa). Using Ni$^{2+}$-chelation chromatography, we were able to partially purify this polypeptide from the bacterial lysate and unequivocally measure 2-O-specific sulfatase activity using the trisulfated, unsaturated heparin disaccharide $\Delta U_{2S}H_{NS,SS}$ as a substrate.

Both the total and soluble protein expression levels achieved were unsatisfactory, however, especially given our previous successes with recombinantly expressing other HSAG-degrading enzymes cloned from F. heparinum (1, 29, 30). As has been the case for most of these enzymes, removal of their putative N-terminal signal sequences greatly facilitated the recombinant expression of soluble protein without compromising their respective specific activities. We likewise identified a putative signal sequence for the flavobacterial 2-O-sulfatase comprised of the first 24 amino acids (see Fig. 2). By engineering a 2-O-sulfatase N-terminal truncation lacking this sequence (herein referred to as 2-O Δ$^{-24}$), we achieved high expression levels of soluble, highly active enzyme. Protein yields exceeding 100 mg of relatively pure sulfatase per liter of induced bacterial cultures were routinely achieved using a single chromatographic step (Fig. 3). The specific activity of the recombinant sulfatase was considerably enhanced following the removal of the N-terminal 6× histidine tag by thrombin cleavage. Removal of this purification tag resulted in a greater than 10-fold purification of sulfatase activity relative to the crude bacterial lysate (Table II). For this reason, we used the cleaved protein in all subsequent experiments. The molecular mass of this recombinantly expressed sulfatase as determined by MALDI mass spectrometry is 50,120.8 daltons. This empirical value closely agrees with its theoretical mass of 49,796 daltons, which is based entirely on its amino acid composition.

To establish the recombinant enzyme’s exclusivity for the uronic acid 2-O-sulfate, we initially compared two related unsaturated heparin disaccharides: $\Delta U_{2S}H_{NS,SS}$ versus $\Delta U_{H_{NS,SS}}$. The recombinant sulfatase only hydrolyzed a single sulfate, namely the one found at the 2-OH-position (Fig. 5A). Other sulfated positions were not hydrolyzed (data not shown).

Biochemical Conditions for Optimal in Vitro Activity—Having successfully achieved the recombinant expression and purification of the flavobacterial sulfatase as a soluble enzyme as well as demonstration of its unequivocal specificity for the uronic acid 2-O-sulfate, we next set out to define the reaction conditions required for optimal enzyme activity in vitro. These parameters included pH, temperature, ionic strength, and possible divalent metal ion dependence. In brief, the enzyme exhibited a pH activity range between 6.0 and 7.0, with optimum activity occurring at pH 6.5 (Fig. 4A). The enzyme was essentially inactive at the outlying pH values of 5.0 and 8.0. In terms of different buffer systems (all at pH 6.5), an imidazole-based buffer demonstrated the highest relative activity as compared

---

**FIG. 1.** Flavobacterium 2-O-sulfatase purification and proteolysis. A, final RP-HPLC chromatography of blue Sepharose CL-6B-purified sulfatase. B, C-4 RP-HPLC chromatographic resolution of sulfatase peptides generated by a trypsin digestion of the major peak shown in A.

**TABLE I** 2-O-sulfatase peptides and corresponding degenerate primers

| Peak No. | Peptide sequence     | Degenerate primers                  |
|----------|----------------------|-------------------------------------|
| 2        | YIVYDKGEIR           | 5‘-TAYATHGTNTAYGAYAARGG-3’           |
| 3        | TYPSVGWNESQWR        | 5‘-NCCYTRTCTRANACDATRTA-3’           |
| 4        | KMPHEFTGFTGNTPEKDGWPSVLMGK | 5‘-CARTGCYGGNTTYGARACNAT-3’         |
| 5        | VAQHGFEITENTMGIMGYTDATVPSQCANFNK | 5‘-DATNGTYTATTNCCRTGYTG-3’      |
| 8        | TDDQVLVCNGIDHPTICGFAI AK | 5‘-NCCYTRTCTRANACDATRTA-3’           |
with buffers containing 50 mM MES, ADA, or phosphate. As expected, phosphate buffer was clearly inhibitory (see histogram, Fig. 4A, inset).

We also examined 2-O-sulfatase activity relative to ionic composition. The recombinant enzyme was optimally active at \( \frac{1}{2} \) 50 mM NaCl. Activity was sharply inhibited by [NaCl] exceeding 100 mM, with 50% inhibition occurring at less than 250 mM NaCl (Fig. 4B). Maximal enzyme activity was largely unaffected by the addition of EDTA up to a 1 mM concentration. The addition of exogenous CaCl₂, MgCl₂, or MnCl₂ (up to 10 mM) also had no substantive effect, indicating that these particular divalent metal ions are not required (data not shown). A preincubation of the enzyme with 5 mM EDTA did result in an \( \frac{1}{2} \) 10% inhibition of activity using the trisulfated disaccharide as a substrate.

37 °C was the default temperature at which all of the preliminary biochemical experiments were conducted. We measured both relative enzyme activity and stability as a function of...
various heparin disaccharides were used, each with a sulfation within the glucosamine. In addition, the two bond at the nonreducing end but differing in the degree of sulfation on the adjoining hexosamine; 2) the trisulfated chondroitin disaccharide (DiS) $\Delta U_{2S}\text{GalNAc}$ was clearly the preferred substrate, whereas the monosulfated disaccharide (DiS) $\Delta U_{2S}\text{HNS}$ was the least preferred substrate. The disulfated disaccharides $\Delta U_{2S}\text{HNS}$ and $\Delta U_{3S}\text{HNS}$ had $k_{cat}/K_m$ values intermediate between these two extremes.

The 2-O-sulfated chondroitin disaccharide $\Delta U_{2S}\text{GalNAc}$ was only negligibly hydrolyzed under the same kinetic conditions. The enzyme did desulfate this disaccharide to an appreciable extent, however, under reaction conditions involving a 4 times higher enzyme concentration and a longer incubation time (data not shown). Under these conditions, ~40% of the substrate was desulfated over a 20-min period. In contrast, less than 10% of chondroitin disaccharide $\Delta U_{2S}\text{GalNAc}$ was hydrolyzed during the same time period. To determine whether either or both of these 2-O-sulfated chondroitin disaccharides...
Cloning and Expression of the Flavobacterial 2-O-Sulfatase and the Δ4,5-Glycuronidase. A 2 mM concentration of the unsaturated, 2-O-sulfated heparin disaccharide ΔU₂SH₂H₂NS₆S was preincubated with either 250 nM Δ4,5-glycuronidase or 25 nM 2-O-ΔN₁₋₂₄ for 2 min at 30 °C in a 100-μl reaction. Following this preincubution, the reciprocal enzyme was added to the reaction for up to an extra 6 min. Δ4,5-glycuronidase activity was measured in real time as the rate of substrate disappearance monitored by the loss of UV absorption at 232 nm. Zero time on the x axis represents the time following the preincubation, during which the second enzyme was added.

**TABLE III**

2-O-sulfatase disaccharide substrate specificity

| Disaccharide | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}/K_m\) |
|--------------|----------------|---------|------------------|
| ΔU₂SH₂H₂NS₆S | 1672          | 0.515   | 3247             |
| ΔU₂SH₂H₆S₂₆S | 814           | 0.087   | 9356             |
| ΔU₂SH₂H₆S₁₄S | 911           | 1.06    | 859              |
| ΔU₂SH₂H₂G₆S₁₄S | 673         | 4.66    | 144              |
| ΔU₂SH₂GalNAc₆S₁₄S | <100         | >10     | ND*              |

* Kinetic values for the unsaturated chondroitin disaccharides were approximated from double reciprocal plots (data not shown). ND, not determined.

could be quantitatively desulfated under exhaustive conditions, we carried out an 18-h incubation at 30 °C that included 5 mM substrate and 5 μM enzyme. Under these conditions, both chondroitin disaccharides were greater than 95% desulfated at the 2-O-position (data not shown). This result indicates that whereas linkage position and/or hexosamine isomerization are discriminating kinetic factors, these physical parameters are not absolute determinants for 2-O-sulfatase substrate recognition.

The Substrate-Product Relationship between the 2-O-Sulfatase and Δ4,5-Glycuronidase—As we have already noted, the flavobacterial Δ4,5-glycuronidase is unable to hydrolyze unsaturated saccharides possessing a uronic acid 2-O-sulfate at the nonreducing end (1). Considering this fact, an obligatory substrate-product relationship between the 2-O-sulfatase and the Δ4,5-glycuronidase must apparently exist. We examined a possible kinetic relationship between these two enzymes by looking at their sequential action (Fig. 6). In this experiment, Δ4,5-glycuronidase activity was measured directly either during or following the addition of the recombinant 2-O-sulfatase using the disaccharide substrate ΔU₂SH₂H₂NS₆S. As expected, when this disaccharide was incubated with the Δ4,5 enzyme alone, it was completely refractory to glycuronidase-mediated hydrolysis as measured by a loss of absorbance at 232 nm. A 2-min preincubation of the substrate with the 2-O-sulfatase, however, resulted in robust linear glycuronidase activity. This rate was comparable with the rate of hydrolysis measured for the control substrate ΔU₂SH₂H₂NS using the Δ4,5 enzyme alone (data not shown). In the reciprocal experiment (i.e. in which the 2-O-sulfatase was added second), we observed an initial lag in Δ4,5 activity. This lag was followed by a linear Δ4,5 activity, albeit at a slower rate than in the case where the 2-O-sulfatase was added first. The observed delay in activity was presumably due to the prerequisite 2-O-desulfation of the substrate, which must occur prior to being acted on by the glycuronidase. This experiment clearly demonstrates at least a functional linkage between these two HSGAG-degrading enzymes. We were unable to show a kinetic coupling of these two enzymes, however, in a related experiment where 2-O-sulfatase activity was measured directly using the same disulfated disaccharide (ΔU₂SH₂H₂NS₆S) substrate in the absence versus the presence of the Δ4,5-glycuronidase (data not shown).

With the results just described, we considered the parallel use of these two enzymes (along with the heparinases) as complementary tools for HSGAG compositional analyses. The utility of this combinatorial approach is shown in Fig. 7. 200 μg of heparin were first subjected to an exhaustive heparinase treatment. Subsequent treatment of the cleavage products with the Δ4,5-glycuronidase resulted in the disappearance of select saccharide peaks, namely those that did not possess a 2-O-sulfated uronic acid at the nonreducing end (Fig. 7B). Con-
versely, subsequent treatment of the heparinase-derived saccharides with the 2-O-sulfatase results in both the disappearance of 2-O-sulfated disaccharides and a concomitant appearance of their desulfated products (Fig. 7C). When both the Δ4,5-glycuronidase and the 2-O-sulfatase were added simultaneously to the heparinase cleavage products, essentially all of the saccharides were hydrolyzed by the Δ4,5-glycuronidase, as evident by a lack of any UV-absorbable electrophoresis products (Fig. 7D).

**DISCUSSION**

Heparin and related heparan sulfate are structurally complex glycosaminoglycans. Their extensive chemical heterogeneity plays out in a functional context, namely in dictating how these linear oligosaccharides mediate a diverse number of biological signaling pathways. One of the more formidable challenges currently facing the glycochemistry field is the design of effective analytical methods to study this structure-function relationship at the molecular level. Structure-specific enzymatic tools are indispensable toward meeting this challenge. The utility of HSGAG-degrading enzymes as such has been considered for quite a long time. The heparin lyases (heparinases) from *F. heparinum* are an excellent example of how such enzymes have been put to such a use. However, whereas the heparinases may be an important prototype, additional HSGAG-degrading enzymes with more defined substrate specificities are now required to refine present methods for sequencing these complex carbohydrates. Toward this end, we have recently cloned from the flavobacterial genome an unsaturated HSGAG glycuronidase (1) with a rather unusual substrate specificity (31). The Δ4,5-glycuronidase can be recombinantly expressed in *E. coli* as a soluble, highly active enzyme. We have shown how this readily purified enzyme can be used as an enzymatic tool to refine our property-encoded nomenclature-MALDI-based method for sequencing heparinase-derived HSGAG oligosaccharides (21). In this paper, we describe the molecular cloning, recombinant expression, and biochemical characterization of another highly useful flavobacterial HSGAG-degrading enzyme, the 2-O-sulfatase.

The cloned, full-length gene encodes an open reading frame of 468 amino acids (Fig. 2), with a predicted molecular mass of 51.9 kDa. This theoretical molecular weight is ~10,000 less than the value reported in the literature (22). Based on its amino acid composition, the encoded protein is quite basic (theoretical pI of 8.75). A further analysis of its primary amino acid sequence unequivocally places this open reading frame as a member of a larger sulfatase family. Members of this superfamily all possess a defining sulfatase domain that resides within the amino terminus and includes the signature motif (C/S)XpXRXXXS/TIG (25). Demarcating this consensus sequence is the invariant active site cysteine or serine that is covalently modified as a formylglycine. From our sequence alignment, we predict cysteine at position 82 to be, in fact, the covalently modified residue. An empirical demonstration of this active-site aldehyde at this position is made in the following paper (40).

Whereas the cloned flavobacterial sulfatase exhibits the highest sequence similarity to the bacterial arylsulfatases (especially the arylsulfatase from *Pseudomonas aeruginosa*), we point out that a limited homology of the 2-O-sulfatase does extend to the mammalian glycosaminoglycan sulfatases functioning in the lysosomal degradation pathway (see Fig. 1 in the accompanying paper (40)). As is the case for the bacterial enzymes, this sequence homology is strongest in the NH2 terminus, where the putative sulfatase domain resides. Among the human lysosomal enzymes, it is the galactosamine (N-acetyl)-6-sulfate sulfatase (chondroitin 6-O-sulfatase) that exhibits the closest similarity with the flavobacterial 2-O-sulfatase; the two enzymes possess ~26% identity when comparing their entire protein sequences. There are also two functionally related lysosomal sulfatases, which specifically hydrolyze the 2-OH-position of uronic acid. These enzymes are the iduronate-2-sulfate sulfatase (32) and the glucuronic 2-sulfate sulfatase (33). The iduronate-2-sulfate sulfatase and flavobacterial 2-O-sulfatase exhibit only a limited sequence homology (less than 22% identity), however.

Like the Δ4,5-glycuronidase, we successfully expressed the 2-O-sulfatase in *E. coli*, from which several milligrams of highly active, soluble enzyme were readily purified. As was also the case for the glycuronidase, we found that the yield of soluble recombinant enzyme was greatly improved by the engineered removal of the hydrophobic N-terminal signal sequence composed of the first 24 amino acids. This signal sequence was predicted by the Von Heinje method, which also identified the likely signal peptidase cleavage recognition sequence AXAXA. With only one exception, we have identified a
putative signal sequence in all of the flavobacterial HSGAG-degrading enzymes examined to date. The general presence of this tag, therefore, would suggest a common periplasmic locale within the flavobacterium for glycosaminoglycan degradation.

Our initial assessment of 2-O-sulfatase activity was based upon the use of a few select unsaturated heparin disaccharide substrates. Desulfation was unequivocally specific for the 2-O-position (Fig. 5A). Our examination of the biochemical conditions for optimal enzymatic activity yielded several observations. First, 2-O-sulfatase activity exhibited a pH profile generally in agreement with previously published results (22), albeit with a narrower pH range (6.0–7.0) in which the enzyme was most active. The enzyme exhibited maximal catalytic efficiency at pH 6.5 with essentially no activity observed at the outlying pH values of 5 and 8 (Fig. 4A). A sharply defined pH optima of 6.5 clearly implicates a catalytic role of one or more histidines. Of the 8 histidines present in the flavobacterial 2-O-sulfatase, His-136 and His-191 are highly conserved among the sulfatases examined. Catalytically important histidines have been observed within the active site of several sulfatase crystal structures including human lysosomal N-acetylglucosaminylsulfatase-4-sulfatase (arylsulfatase B) (27) and arylsulfatase A (25) as well as the arylsulfatase from P. aeruginosa (34) to which the flavobacterial 2-O-sulfatase appears to be most closely related. We have in fact used the structures of these related enzymes to construct a highly informative homology model for the flavobacterial 2-O-sulfatase. This model and its structure-function implications are presented in the accompanying paper (40).

Second, the observed NaCl titration profile (Fig. 4B) demonstrates a clearly inhibitory effect of ionic strength on sulfatase activity, even at relatively low NaCl concentrations. In other words, whereas 50% inhibition occurred in the presence of ~200 mM NaCl, even 100 mM NaCl was slightly inhibitory to 2-O-sulfatase activity. We have likewise noted this rather sharp activity transition for both the Δ4,5-glycuronidase and other recombinantly expressed F. hepiorum GAG-degrading enzymes. This correlation between activity and the ionic buffer composition is not surprising, given the anionic character of the saccharide substrates conferred by both the presence of sulfates and the uronic acid carboxylates within each disaccharide unit. For the 2-O-sulfatase in particular, charge interactions between basic side chains and the sulfate oxygen anion would be clearly important for substrate orientation. Additional interactions with the uronic acid C-5 carboxylate are also likely to occur. A masking of these important charges by exogenous ions would logically interfere with their catalytic function.

The flavobacterial 2-O-sulfatase also possesses 52 acidic amino acids, at least three of which are highly conserved (e.g. Asp-42, Asp-63, and Asp-295). Four acidic side chains are also found in a consensus active site derived from known crystal structures. In this snapshot, these four carboxylates appear to in vivo inhibit the flavobacterial 2-O-sulfatase activity, even at relatively low NaCl concentrations. In other words, whereas 50% inhibition occurred in the presence of ~200 mM NaCl, even 100 mM NaCl was slightly inhibitory to 2-O-sulfatase activity. We have likewise noted this rather sharp activity transition for both the Δ4,5-glycuronidase and other recombinantly expressed F. hepiorum GAG-degrading enzymes. This correlation between activity and the ionic buffer composition is not surprising, given the anionic character of the saccharide substrates conferred by both the presence of sulfates and the uronic acid carboxylates within each disaccharide unit. For the 2-O-sulfatase in particular, charge interactions between basic side chains and the sulfate oxygen anion would be clearly important for substrate orientation. Additional interactions with the uronic acid C-5 carboxylate are also likely to occur. A masking of these important charges by exogenous ions would logically interfere with their catalytic function.

The flavobacterial 2-O-sulfatase also possesses 52 acidic amino acids, at least three of which are highly conserved (e.g. Asp-42, Asp-63, and Asp-295). Four acidic side chains are also found in a consensus active site derived from known crystal structures. In this snapshot, these four carboxylates appear to coordinate a divalent metal ion (typically calcium). We could find no empirical evidence, however, for the requirement of divalent metal ions for maximal sulfatase activity. The enzyme was largely unaffected by either a preincubation with EDTA (up to 5 mM) or the addition of Ca²⁺, Mg²⁺, or Ni²⁺. We were somewhat surprised by this in vitro observation given the structural inference of a common metal binding site and a report in the literature describing inactivation of the glucuronate-2-sulfatase with EDTA (35). Whereas this may be so, not all sulfatasdes described to date have been shown in fact to be inactivated by EDTA. The issue of metal ion chelation is discussed in greater detail in the paper that follows (40).

As stated earlier, the recombinant flavobacterial 2-O-sulfatase acted on every unsaturated heparin disaccharide examined, provided each saccharide possessed a 2-O-sulfated uronic acid at its nonreducing end. We were particularly interested, however, in ascertaining any kinetic discrimination the enzyme may possess for its substrates based on additional structural considerations, namely the number and position of sulfates on the adjoining hexosamine, glycosidic linkage position (i.e. β1–4 versus α1–3) and the 2-OH-position of the anomeric carbon within this hexosamine (i.e. glucosamine versus galactosamine). For the 2-O-sulfated, unsaturated heparin disaccharides examined, the enzyme exhibited $k_{cat}$ and $K_m$ values roughly in the range of 600–1700 s⁻¹ and 0.1–1 mM, respectively. Both of these parameters generally agree with values described in earlier studies using the sulfatase directly purified from flavobacterial lysates. In directly comparing the four heparin disaccharide substrates tested, however, we noted a clear kinetic discrimination exhibited by the recombinant enzyme; one based on the extent of sulfation and largely manifested as a $K_m$ effect. In particular, it appears that substrate binding is most favorably conferred by the presence of a sulfate at the 6-OH-position of the adjoining glucosamine. Sulfation of the amine likewise confers a positive effect, albeit to a somewhat lesser degree relative to the 6-OH-position.

Interestingly, the 2-O-sulfatase was also able to desulfate unsaturated chondroitin disaccharides at the 2-OH-position. The recombinant enzyme is, therefore, both a heparin/heparan and chondroitin 2-O-sulfatase. Compared with the corresponding heparin substrate, the rate of hydrolysis was markedly slower, however (Fig. 5B). This result indicates that whereas linkage position and/or hexosamine isomerization are discriminating kinetic factors, these physical parameters are not absolute determinants for 2-O-sulfatase substrate recognition. It is interesting to consider this latter observation in the context of the lysosomal pathway for glycosaminoglycan degradation in mammals where one enzyme desulfates both chondroitin and HS oligosaccharides at this position.

In our studies, we exclusively used unsaturated saccharide substrates on the presumption that they are the naturally occurring substrate in vivo. If the 2-O-sulfatase ordinarily acts on heparinase-derived saccharides in a sequential HSGAG degradation pathway, we can also place the activity of the 2-O-sulfatase “upstream” from the hydrolysis of the unsaturated uronic acid by the Δ4,5-glycuronidase. The obligatory substrate-product relationship between the 2-O-sulfatase and the Δ4,5-glycuronidase has been described by our laboratory (1) and others (36). In this paper, we further demonstrate this correlation in experiments summarized in Figs. 6 and 7. Fig. 7 also illustrates how these two enzymes (along with the heparinases) can be used in tandem as analytical tools for HSGAG compositional analyses.

As we pointed out earlier, there are 2-O-sulfatasdes present in the mammalian glycosaminoglycan degradation pathway. Whereas both of these enzymes desulfate heparan sulfate, the idurionate-2-sulfate sulfatase also acts on dermatan sulfate. Both enzymes possess an acidic pH optimum for activity, a fact consistent with their location within the lysosome. The two sulfatasdes initially exist as precursors that must be proteolytically processed for activity. The native molecular mass of the human idurionate-2-sulfate sulfatase precursor has been reported in the range of 42–65 kDa (32), whereas its theoretical mass based entirely on its amino acid composition is ~62 kDa. As such, the mammalian lysosomal idurionate-2-sulfate sulfatase is somewhat larger than its flavobacterial counterpart, while also requiring substantial posttranslational modification for maximal enzyme activity. The acidic pH optima for the lysosomal enzymes would also appear to limit their in vitro use for the determination of HSGAG composition, at least when
used in tandem with other flavobacterial HSGAG-degrading enzymes such as the heparinas or the Δ4,5-glycuronidase; these latter enzymes all possess a pH optimum much closer to neutrality.

The obvious fact that we are able to measure robust, 2-O-specific sulfatase activity for the recombinantly expressed enzyme argues, at least from a functional perspective, for the presence of the critical catalytic formylglycine within the sulfatase active site. This functionality clearly validates our use of the E. coli genome. The heterologous expression of other catalytically active sulfatasases in E. coli has been reported (34, 37). One notable exception of which we are aware is the mucin-desulfating enzyme from Prevotella sp. (38). Interestingly, this particular desulfating enzyme possesses a modified serine rather than a cysteine within its active site, a fact supported by our own multiple sequence alignment (see Fig. 1 in the accompanying paper (40)). To the best of our knowledge, nearly every active, recombinantly expressed sulfatase reported in the literature possesses a cysteine (and not a serine) within the active site sequence (C/S)X(P/R)XXX(S/T)G. It seems likely, therefore, that a cysteine-specific modifying machinery functionally exists in E. coli. Our initial attempts to produce a recombinant cysteine → serine 2-O-sulfatase variant led to the production of insoluble protein when expressed in E. coli. Consequently, we are currently unable to empirically address this hypothesis using the 2-O-sulfatase as a prototype. We point out the curious fact that the E. coli genome encodes for at least three different putative sulfatase genes, in addition to the atsB gene, which, by homology, has been proposed to encode for this cysteine-specific modifying activity. All of these genes are located as a cluster within the bacterial chromosome (39). It would appear, however, that the E. coli sulfatase genes are normally cryptic. At the very least, E. coli lacks the specific enzymes for desulfating heparin/heparan sulfate glycosaminoglycans. In any case, the bacterium fortuitously provides the necessary enzymology to effectively modify select heterologous sulfatases such as the 2-O-sulfatase. We look forward to the use of this facile expression system to recombinantly express other flavobacterial glycosaminoglycan sulfatasases as their respective genes are identified.

Acknowledgments—We thank Kevin Pojasek and David Eaverone for a critical reading of the manuscript. We also thank Joseph Davis for technical assistance.

REFERENCES
1. Myette, J. R., Shriver, Z., Knizlpepe, T., McLean, M. W., Venkataraman, G., and Sasisekharan, R. (2002) Biochemistry 41, 7424–7434
2. Erko, J. D., and Lindahl, U. (2001) J. Clin. Invest. 108, 169–173
3. Shriver, Z., Liu, D., and Sasisekharan, R. (2002) Trends Cardiovasc. Med. 12, 71–77
4. Perrimon, N., and Bernfield, M. (2000) Nature 404, 725–728
5. Simeon, A., Wegrowsky, Y., Bontemps, Y., and Maquart, F. X. (2000) J. Invest. Dermatol. 115, 962–969
6. Ishikawa, Y., and Kitamura, M. (1999) Kidney Int. 56, 954–963
7. Kapila, Y. L., Wang, S., Dazin, P., Taffola, E., and Mass, M. J. (2002) J. Biol. Chem. 277, 8482–8491
8. Selva, E. M., and Perrimon, N. (2001) Adv. Cancer Res. 83, 67–80
9. Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanaswami, U. (2002) Nat. Rev. Cancer 2, 521–528
10. Polkman, J., and Shing, Y. (1992) Adv. Exp. Med. Biol. 313, 355–364
11. Vladarsky, I., Elkin, M., Pappo, O., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Avir, A., Peeker, I., and Friedman, Y. (2000) Isr. Med. Assoc. J. 2, (suppl.) 37–45
12. Coldberg, J. A., Li, J., Uversky, V. N., and Fink, A. L. (2002) Biochemistry 41, 1592–1591
13. Schayek, E., Olivercova, T., Bengtsson-Olivercova, G., Vladovska, I., Levkovitz, H., Ayver, R., and Eisenberg, S. (1995) Atherosclerosis 114, 1–8
14. Liu, J., and Thorp, S. C. (2002) Med. Res. Rev. 22, 1–25
15. Tumova, S., Wunds, A., and Couchman, J. R. (2000) Int. J. Biochem. Cell Biol. 32, 269–288
16. Lyon, M., and Gallacher, J. T. (1998) Matrix Biol. 17, 485–493
17. Gallagher, J. T. (1997) Biochem. Soc. Trans. 25, 1206–1209
18. Ernst, S., Langer, R., Cooney, C. L., and Sasisekharan, R. (1995) Crit. Rev. Biochem. Mol Biol 30, 387–444
19. Homberg, A. J., Ernst, S., Sasisekharan, R., and Biemann, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4176–4181
20. Sasisekharan, R., Bulmner, M., Moremen, K. W., Cooney, C. L., and Langer, R. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 3660–3664
21. Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R. (1999) Science 286, 531–542
22. McLean, M. W., Bruce, J. S., Long, W. F., and Williamson, F. B. (1984) Eur. J. Biochem. 145, 607–615
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1987) Current Protocols in Molecular Biology, Vol. 1, pp. 6.1.1–6.4.2, John Wiley and Sons, Inc., New York
24. Nielsen, H., Engelbrecht, J., Brunak, S., and von Hejne, G. (1997) Protein Eng. 10, 1–6
25. Lukatelis, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K., and Saenger, W. (1998) Biochemistry 37, 3654–3664
26. Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. (1995) Cell 82, 271–278
27. Bond, C. S., Clements, P. R., Ashby, S. J., Collyer, C. A., Harrop, S. J., Hopwood, J. J., and Guss, J. M. (1997) Structure 5, 277–289
28. Parenti, G., Meroni, G., and Ballabio, A. (1995)Curr. Opin. Genet. Dev. 7, 386–391
29. Ernst, S., Venkataraman, G., Winkler, S., Fedtke, S., Raman, R., and Sasisekharan, R. (1998) Biochem. J. 315, 589–597
30. Pojasek, K., Shriver, Z., Kiley, P., Venkataraman, G., and Sasisekharan, R. (2001) Biochem. Biophys. Res. Commun. 286, 343–351
31. Warnick, C. T., and Linker, A. (1972) Biochemistry 11, 568–572
32. Bielicki, J., Freeman, C., Clements, P. R., and Hopwood, J. J. (1990) Biochem. J. 271, 75–86
33. Freeman, C., and Hopwood, J. J. (1989) Biochem. J. 259, 209–218
34. Boltes, I., Czapinska, H., Kahnert, A., von Bulow, R., Durka, T., Schmidt, B., von Figura, K., Kertesz, M. A., and Uson, I. (2001) Structure (Camb.) 9, 483–491
35. Freeman, C., and Hopwood, J. J. (1991) Biochem. J. 279, 399–405
36. Dietrich, C. P., Silva, M. E., and Michelacci, Y. M. (1973) J. Biol. Chem. 248, 6408–6415
37. Durka, T., Miech, C., Hummerrer-Johnn, H., Schmidt, B., Kertesz, M. A., and von Figura, R. (1998) J. Biol. Chem. 273, 25560–25564
38. Wright, D. P., Knight, C. G., Parker, S. G., Christie, D. L., and Robertson, A. M. (2000) J. Bacteriol. 182, 3002–3007
39. Kertesz, M. A. (2000) FEMS Microbiol. Rev. 24, 135–175
40. Raman, R., Myette, J. R., Shriver, Z., Pojasek, K., Venkataraman, G., and Sasisekharan, R. (2003) J. Biol. Chem. 278, 12167–12174