Organization of the Inter-α-Inhibitor Heavy Chains on the Chondroitin Sulfate Originating from Ser_{10} of Bikunin: Posttranslational Modification of IαI-Derived Bikunin†

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ABSTRACT: Inter-α-inhibitor-derived bikunin was purified and the molecular mass was determined to be ~8.7 kDa higher than the prediction based on the protein sequence, suggesting extensive posttranslational modifications. These modifications were identified and characterized by a combination of protein and carbohydrate analytical techniques. Three modifications were identified: (i) glycosylation of Ser_{10}, (ii) glycosylation of Asn_{45}, and (iii) a heterogeneous truncation of the C-terminus. The Asn_{45} associated glycan was shown to be a homogenous "complex type" biantennary structure. The chondroitin-4-sulfate (CS) chain attached to Ser_{10} was analyzed by both matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and acrylamide gel electrophoresis after partial chondroitin ABC lyase digestion. The analyses showed that the CS chains were composed of 15 ± 3 [GlcUA-GalNAc] disaccharide units. On average, every forth disaccharide was sulfated, and these sulfated disaccharides appeared to be more common near the reducing end. Anion exchange chromatography at pH 3.4 of intact bikunin resulted in the isolation of four isotypes shown to differ only in the amount of sulfation. Heavy chain 1 (HC1) and heavy chain 2 (HC2) are attached to the CS by a novel cross-link [Enghild, J. J., Salvesen, G., Hefta, S. A., Thøgersen, I. B., Rutherfurd, S., and Pizzo, S. V. (1991) *J. Biol. Chem. 266*, 747−751], and the order in which the two heavy chains are positioned on the CS was examined. The results indicate that HC1 is in close proximity to HC2 and both are near the less sulfated nonreducing end of the CS. Taken together, the data show the following organization of the IαI molecule: $[GlcUA\text{-}GalNAc]_a$-HC1-$[GlcUA\text{-}GalNAc]_b$-HC2-$[GlcUA\text{-}GalNAc]_c$-Gal-Gal-Xyl-Ser_{10}-bikunin, ($a + b + c = 12-18$ disaccharides).

Most proteases in human blood belong to the chymotrypsin serine protease family. They usually circulate as zymogens and are activated as needed during coagulation, fibrinolysis, complement activation, and inflammation. The active enzymes are controlled by an abundance of protease inhibitors accounting for about 10%, by weight, of all proteins in the blood (*1*). Although approximately 20 serine protease inhibitor families have been classified (*2*), most of the serine protease inhibitors identified in human blood belong to either the bovine pancreatic trypsin inhibitor (Kunitz) or "kunin" family (*3*−*5*) or the serpin family (*6*). Blood kunins include tissue factor pathway inhibitor 1 and 2 (*7*, *8*), amyloid precursor protein and amyloid precursor protein homologue (*9*, *10*), hepatocyte growth factor activator inhibitor (*11*), inter-α-inhibitor (IαI),[1] pre-α-inhibitor (PαI), and HC2 HC2·bikunin (*12*) (see refs *13* and *14* for recent reviews). Another human kunin is placental bikunin (*15*, *16*).

The multichain bikunin proteins, IαI, PαI, and HC2·bikunin, are by far the most abundant kunins in blood (450 mg/L) (*17*)). Free bikunin is found in urine and has been called urinary trypsin inhibitor (UTI) (*18*, *19*). IαI (225 kDa) is a glycoprotein composed of bikunin, heavy chain 1 (HC1), and heavy chain 2 (HC2). PαI (125 kDa) is composed of bikunin and heavy chain 3 (HC3), and HC2·bikunin (125 kDa) is composed of bikunin and HC2 (*12*). The three heavy chains are homologous but distinct gene products (*20*−*23*). The heavy chains show identity to another plasma protein,

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfate; CS, chondroitin sulfate; FACE, fluorophore-assisted carbohydrate electrophoresis; Gal, galactose; GalNAc, *N*-acetylhexosamine; GlcUA, glucuronic acid; HA, hyaluronic acid; HexUA, hexuronic acid; HPLC, high performance liquid chromatography; IαI, inter-α-inhibitor (IαI); IHRP, inter-α-trypsin inhibitor family heavy chain related protein; Lys-C, endoproteinase Lys-C; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PαI, pre-α-inhibitor; PGP, protein glycosaminoglycan protein cross-link; PVDF, polyvinylidene difluoride; Spase V8, *Staphylococcus aureus* V8 protease; Xyl, xylose.

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inter-α-trypsin inhibitor family heavy chain related protein (IHRP). Interestingly, IHRP is not bound to bikunin (*24*, *25*). Bikunin is encoded for by the α_1-microglobulin-bikunin gene (*26*). However, during biosynthesis the precursor protein is proteolytically processed and the released free bikunin and the heavy chains are assembled intracellularly before secretion (*27*, *28*). A large fraction of the free bikunin escapes assemble and is probably the origin of the free bikunin found in urine (*28*).

The multichain bikunin proteins, IαI, PαI, and HC2• bikunin, resist dissociation in reduced SDS−PAGE but can be dissociated by chondroitin-4-sulfate (CS) degrading enzymes (*12*, *29*). We have previously described the structure of this novel protein glycosaminoglycan protein (PGP) cross-link (*30*). The heavy chains are covalently bound to the bikunin Ser_{10} CS via an ester bond between the α-carbon of the C-terminal Asp and carbon-6 of an internal *N*-acetyl-galactosamine of the CS chain. This description was the first of a glycosaminoglycan cross-linking polypeptide chains. The PGP cross-link has subsequently been found in all multichain members of the bikunin proteins (*31*, *32*).

In addition to the structural role of the PGP cross-link, the bikunin CS may directly mediate biological processes outside the vascular space (*33*). For example, plasma IαI may regulate oocyte maturation by binding to the hyaluronic acid (HA) rich extracellular matrix of the oocyte−cumulus cell complex, thereby limiting the expansion of this complex (*34*−*36*). In fact, heavy chains of the bikunin proteins may bind covalently to HA by displacing bikunin (*37*). Additionally, the antiinflammatory TSG-6 glycoprotein (*38*), found in synovial fluid of rheumatoid arthritis patients, binds covalently to IαI in a glycosaminoglycan-dependent manner (*39*).

The interaction of the bikunin proteins with components of the extracellular matrix may result in the apparently spontaneous dissociation and displacement of heavy chains and bikunin and the concurrent incorporation of other proteins or HA (*34*−*37*, *39*). Neither the known molecular structure of the PGP cross-link nor the nature of CS readily explains these observations, and a more detailed description of the overall "architecture" of the bikunin CS component is a prerequisite for understanding the molecular mechanisms behind these observations. We present an analysis and characterization of the CS and other posttranslational modifications of bikunin.

MATERIALS AND METHODS

Materials. Staphylococcus aureus V8 protease (SPase V8) (EC 3.4.21.19), neuraminidase (EC 3.2.1.18), β-galactosidase (EC 3.2.1.23), *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), endo-glycosidase F (EC 3.2.1.96), and *N*-glycosidase-F (EC 3.2.2.18) were obtained from Boehringer Mannheim (Mannheim, Germany). Endoproteinase Lys-C (EC 3.4.21.50) (Lys-C) was from Wako (Richmond, VA). Chondroitin ABC lyase (EC 4.2.2.4), unsaturated disaccharide standards, and chondroitin were from Seikagaku (Tokyo, Japan). Reagents and buffers for fluorophore-assisted carbohydrate electrophoresis (FACE) were supplied by Glyko Inc. (Novato, CA). 8-Aminonaphthalene-1,3,6-trisulfate (ANTS) was from Glyko Inc. (Novato, CA) or Molecular Probes (Eugene, OR). IαI was purified as previously described (*12*).

Purification of IαI-Derived Bikunin. IαI was treated with 50 mM NaOH for 15 min (*30*). This procedure dissociates IαI without any detectable protein degradation. Bikunin was purified by reversed-phase HPLC using a 2.1 cm × 22 cm Aquapore RP-300 column (Brownlee) connected to an ABI 130A HPLC system. The column was equilibrated in 0.1% TFA and developed with a gradient to 60% acetonitrile at 2% min^{-1} and a flow rate of 200 μL min^{-1}. The trypsin inhibition counter-staining technique (*12*, *40*) was used to assay the fractions for trypsin inhibitory activity. The bikunin-containing fractions were collected and lyophilized.

SDS−Polyacrylamide Gel Electrophoresis (PAGE). Samples were boiled in SDS sample buffer in the presence of 50 mM dithiothreitol. SDS−PAGE was performed in 5%−15% gradient gels (10 cm × 10 cm × 0.15 cm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described previously (*41*). The gels were stained for protein in the conventional way with use of Coomassie blue or the trypsin inhibition counter-staining technique that specifically visualizes trypsin inhibitory activity (*12*, *40*).

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired with use of a prototype linear time-of-flight mass spectrometer (Applied Biosystems Sweden AB, Uppsala, Sweden) equipped with 0.7 m flight tube, a 337 nm nitrogen laser, and a 300 MHz digitizer. The samples were prepared by the sandwich preparation method and with α-cyano-4-hydroxycinnamic acid as the matrix (*42*). The spectra were calibrated by external calibration if not stated otherwise.

Protein Sequence Analysis and Amino Acid Analysis. Automated Edman degradation was performed on an Applied Biosystems 477A sequencer with on-line phenylthiohydantoin analysis via an Applied Biosystems 120A HPLC system. Samples were prepared by HPLC or by SDS−PAGE, followed by electrotransfer to polyvinylidene difluoride (PVDF) membrane (*43*). Peptide concentrations were determined by amino acid composition analysis. Peptide samples (approximately 500 pmol) were hydrolyzed for 24 h at 110 °C in 6 N HC1 containing 0.1% phenol. The tubes were evacuated and flushed with nitrogen several times before they were sealed under vacuum (*44*). The hydrolysates were analyzed in a Beckman 6300 amino acid analyzer using sodium citrate buffers provided by the manufacturer.

Separation of Bikunin Isomers. IαI-derived bikunin was separated by anion exchange chromatography using a PC3.2/3 Mini Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) connected to a Pharmacia SMART system. The column was equilibrated in 20 mM glycine−HCl and 75 mM NaCl, pH 3.4. Bikunin isomers were monitored at 280 nm and eluted by a linear gradient from 75 to 500 mM NaCl in 20 mM glycine−HCl, pH 3.4, at 100 μL min^{-1}.

Limited SPase V8 Proteolysis of Bikunin. The bikunin peptide bond Glu_{18}−Val_{19} is particularly susceptible to proteolysis by SPase V8, and this property was used to generate an N-terminally truncated form of bikunin. Bikunin was incubated with 0.01 % w/w SPase V8 for 30 min at 23 °C in 50 mM NH_4HCO_3 before the reaction was terminated by lyophylization.

Complete Endoproteinase Lys-C Digestion of Bikunin. Purified bikunin was treated with chondroitin ABC lyase to remove most of the CS chain as described below (the ΔHexUA-GalNAc-GlcUA-Gal-Gal-Xyl- "stub" is left on

Ser_{10}). Bikunin was then reduced for 30 min in 40 mM dithiothreitol, 6 M guanidine−HCl, and 10 mM EDTA, pH 8, and then alkylated by the addition of 4-vinylpyridine to a final concentration of 90 mM. The reaction was continued for 15 min before the protein was desalted on a NAP 5 column (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris−acetate, pH 8.5. The reduced and alkylated bikunin was digested overnight at 37 °C using Lys-C at an enzyme/substrate ratio of 1:100 (w/w). The peptides were purified by reversed-phase HPLC on a Sephasil R18 microbore column (Pharmacia, Uppsala, Sweden) connected to a Pharmacia SMART system. The column was equilibrated with 3% acetonitrile and 0.1% TFA and then developed with a gradient to 70% acetonitrile at 2% min^{-1} and a flow rate at 200 μL min^{-1}. Peptides were monitored at 214 nm and collected manually.

Sequential Glycosidase Digestion Treatment of N-Linked Glycopeptides. Approximately 100−200 pmol of HPLC-purified peptides was lyophilized, resuspended in 10 μL of 50 mM NH_4OAc, and treated sequentially with 5 mU neuraminidase, β-galactosidase, *N*-acetyl-β-D-glucosaminidase, endo-glycosidase F, or *N*-glycosidase F at 23 °C for 12 h.

Chondroitin ABC Lyase Digestion of Bikunin. Chondroitin ABC lyase was allowed to react with bikunin in 50 mM NH_4HCO_3 at 37 °C at an enzyme/substrate ratio of 1:50 (w/w). The reaction was continued for 12 h.

Fluorophore-Assisted Carbohydrate Composition Analysis. To determine the disaccharide composition of the CS, purified bikunin was digested with chondroitin ABC lyase, and the reactions products were injected on an Aquapore RP-300 reversed-phase column (Brownlee Lab, Perkin-Elmer) equilibrated in water. The unsaturated disaccharides, which have a characteristic absorbance at 230 nm, were collected in the initial elution volume and lyophilized repeatedly to remove remnant ammonia from the NH_4HCO_3 buffer. The bikunin-derived disaccharides were labeled with 2-aminoacridone and analyzed in the FACE system.

The composition of isolated disaccharides was determined using the FACE technology (*45*−*49*) (Glyko, Novato, CA). In brief, the released disaccharides were labeled overnight at 37 °C with the uncharged flourophor 2-aminoacridone, according to the manufacturer's directions. The labeled carbohydrate was resolved on a proprietary gel system at 5 °C, and the gels were imaged with an electronic imaging system based on a CCD camera linked to a computer. The acquired image was analyzed with the FACE software densitometer (version 2.3).

Sequence Analysis of CS. CS chains were released from bikunin by β-elimination of the Xyl−Ser_{10} bond. The reducing terminal xylose was labeled with *p*-aminophenol by reductive amination and the adduct was radio-iodinated (*50*). The radio-iodinated labeled CS chains were subjected to gradient PAGE, either untreated or after partial degradation by chondroitin ABC lyase. The ^{125}I *p*-aminophenol-labeled CS was transfer-blotted onto a nylon membrane and subsequently exposed to X-ray film (*50*−*52*). The CS fragments were also analyzed by gel filtration chromatography using a Bio-Gel P-6, as described previously (*50*).

In other experiments the CS was released from bikunin as described above. The ΔHexUA-GalNAc-GlcUA-Gal-Gal-Xyl- "stub" was produced by extensive chondroitin ABC lyase degradation of the bikunin CS, and a CS standard was generated by digesting CS with limited conditions. The samples were derivatized with the flourophor 8-aminonaphthalene-1,3,6-trisulfate (ANTS), as described previously (*46*−*49*). After completion of the labeling reactions, the intact bikunin CS, the bikunin CS "stub," and the standard CS fragments were resolved on 20% acrylamide gels at 5 °C. These gels were prepared according to Laemmli (*53*) but without SDS in the gel buffers. After electrophoresis the gels were analyzed by an electronic imaging system and with the FACE software densitometer (version 2.3), as described above.

Analysis of the Distance between HC1 and HC2. Purified IαI was dialyzed into 10 mM NH_4HCO_3. A 5 μg amount of IαI was incubated with incubated with 0, 6 ng, 60 ng, 0.3 μg, 0.6 μg, 1.2 μg, or 5 μg of chondroitin ABC lyase for 3 h. The reaction products were analyzed by unreduced SDS−PAGE.

Arrangement of HC1 and HC2 on the CS Chain. The linear unbranched nature of CS allows only two possible configurations; either HC1 or HC2 is closest to the reducing end of the CS. It is also possible that IαI exists in a combination of these two configurations. To address this question, we partially digested 500 μg of reduced and *S*-carboxamidomethylated IαI with 0.03 μg of chondroitin ABC lyase for 30 min at 37 °C in 40 mM Tris−HCl, 40 mM NaCl, pH 8, and 40 mM CH_3COONa. After the reaction, the buffer was exchanged by gel filtration chromatography using a NAP-10 column (Amersham Pharmacia Biotech) equilibrated in 10 mM CH_3COONa, pH 6. The peak fraction (∼100 μg) was then labeled with ANTS, essentially as previously described (*46*−*49*). In brief, the peak fraction from the NAP-10 column was concentrated in a SpeedVac System (Savant, Holbrook, NY) to 15−20 μL. The sample was derivatized by adding 20 μL of CH_3COONa, pH 5, containing 0.2 M ANTS and 20 μL of 1 M cyanoborohydride in dimethyl sulfoxide. The reaction was continued for 3 h at 37 °C before excess reagents were removed by three sequential precipitations of 9 volumes −20 °C ethanol to 1 volume sample. After the third precipitation, the pellet was dissolved in 100 μL of 40 mM Tris−HCl, 40 mM NaCl, pH 8, and 40 mM CH_3COONa and digested extensively using 1.2 μg of chondroitin ABC lyase 3 h at 37 °C. The dissociated heavy chains were resolved by reduced SDS−PAGE, and the gel was imaged by an electronic imaging system and analyzed with the FACE software densitometer (version 2.3). In parallel experiments, the labeled heavy chains were analyzed by reduced SDS−PAGE and identified by Edman degradation after transfer to PVDF membranes.

RESULTS

Isolation of IαI-Derived Bikunin. The components of the bikunin proteins were dissociated by mild NaOH treatment (*30*, *54*). Bikunin was purified by reverse-phase HPLC and analyzed by SDS−PAGE followed by trypsin inhibitor counter (TIC) staining (*12*, *40*). This protocol was chosen because free glycosylated bikunin does not stain well with use of conventional protein staining techniques (*30*). Two bands, with apparent molecular masses in SDS−PAGE of approximately 34 and 23 kDa, were visualized (Figure 1). Edman degradation of both bands after transfer to a PVDF

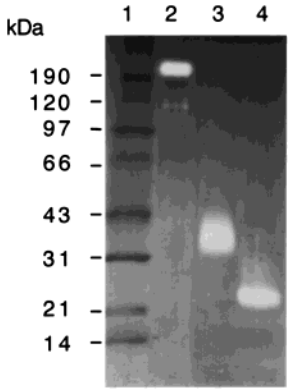

FIGURE 1: Purification of IαI-derived bikunin. IαI was dissociated by gentle NaOH treatment, and the components were separated by reverse-phase HPLC. Size markers (lane 1), purified IαI (lane 2), high molecular mass bikunin (lane 3), and low molecular mass bikunin (lane 4) were analyzed by unreduced SDS−PAGE and stained for trypsin inhibitory activity. Edman degradation of the high molecular mass and low molecular mass bikunin products verified that N-terminal truncation had not taken place.

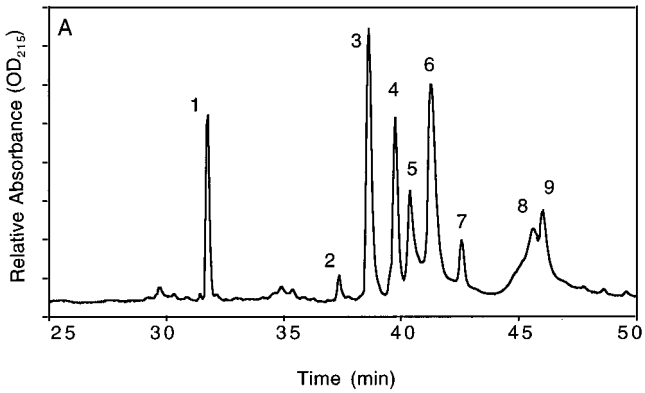

FIGURE 2: Lys-C reversed-phase HPLC peptide map of chondroitin ABC lyase digested bikunin. Reduced and alkylated chondroitin ABC lyase treated bikunin was digested with Lys-C. The peptides derived were separated by HPLC and analyzed by Edman degradation and MALDI-MS (see also Table 1). Peptide 2 contains Ser_{10}, and the attached CS "stub" and peptide 3 contained the Asn_{45} of the N-linked glycan. Peptide 8 is a variant of peptide 9 containing an oxidized Trp residue.

membrane revealed the sequence Ala-Ala-Leu-Pro-Gln-Glu-Glu-Glu-Gly, corresponding to the intact N-terminus of bikunin. We infer that the difference in size between these two species is caused by the release of the CS, presumably by hydrolysis of the Ser_{10}−Xyl glycosidic bond. Matrix-assisted laser desorption/ionization (MALDI-MS) of the two bikunin forms gave the values 18.6 and 24.6 kDa, and these will subsequently be referred to as the "high molecular mass" and "low molecular mass" bikunin forms.

Identification of Posttranslational Modifications. Automated Edman degradation of the N-terminal 20 amino acid residues of bikunin indicated the CS attached to Ser_{10} was the only posttranslational modification. To identify modifications not associated with Ser_{10} the N-terminal 19 residues were removed by limited SPase V8 proteolysis (*30*, *55*), and the bulk of bikunin was purified by HPLC. The expected mass was 14 180.12 Da calculated on the basis of the published cDNA sequence (*26*). The observed *m*/*z* of 15 882 was consistent with the presence of posttranslational modification(s), most likely a glycan attached to Asn_{45}. The mass after N-glycosidase F digestion supported this notion; however, the expected mass of 14 180.12 Da is deviated from the observed *m*/*z* of 13 674 Da probable because of C-terminal truncation.

To examine the posttranslational modifications in detail a Lys-C peptide map of bikunin was generated. To eliminate peptide variants caused by CS polydispersity the CS was truncated by chondroitinase ABC lyase digestion. This procedure leaves the ΔHexUA-GalNAc-GlcUA-Gal-Gal-Xyl "stub" attached to bikunin. The digest was analyzed by HPLC, Edman degradation, and MALDI-MS (Figure 2 and Table 1). Nine fractions were collected (Figure 2) and assigned to the cDNA sequence according to their mass and positions relative to the N-terminus (Table 1). Fractions 1, 3, 5, and 9 were determined to be peptides K7, K6, K8, and K4, respectively. Fractions 4 and 7 contained processed variants of the C-terminal peptide K8. K8 was terminated at Leu_{143} and at Phe_{145}; hence, three forms of the C-terminal peptide terminating at Leu_{143}, Phe_{145}, and Asn_{147} were found. The stoichiometry was estimated at 214 nm during HPLC separation to be 5:1:2.

The N-terminal peptide (K1) containing Ser_{10} was identified in fraction 2. The observed *m*/*z* was 3205.4 and corresponded to the calculated mass of 3202.23 Da including the expected CS "stub" region ΔHexUA-GalNAc(SO_4)-GlcUA-Gal-Gal-Xyl or ΔHexUA-GalNAc-GlcUA-Gal(SO_4)-Gal-Xyl (*56*). Fraction 6 contained the *N*-glycosylated K3 peptide. Sequential exoglycosidase digestion of K3 was used to verify the binantennary complex type glycan at Asn_{45} (Table 2). Monosaccharides were successively liberated from the nonreducing end of the K3 glycopeptide by the action of neuraminidase, *β*-galactosidase, and *N*-acetylhexosaminidase, corresponding, respectively, to the presence of 2 sialic acid, 2 galactose, and 2 *N*-acetylglucosamine saccharides. Surprisingly, the Asn_{45} glycan was found to be homogeneous.

Characterization of the Bikunin CS. The objectives of these analyses were to determine (a) the monosaccharide composition of the CS, (b) the length or sequence of the CS, (c) the relative position of the heavy chains, and (d) the succession or order in which the two heavy chains are attached to the CS.

(a) Composition of CS. The disaccharide composition was examined after digestion with chondroitin ABC lyase. Standard unsaturated disaccharides and bikunin derived unsaturated disaccharides were fluorophore labeled with 2-aminoacridone and analyzed by PAGE (Figure 3). This method separated the standard unsaturated disaccharides, including chondro-di-0-sulfate, chondro-di-4-sulfate, and chondro-di-6-sulfate (Figure 3a). Comparison of the chondroitin ABC lyase digest with the defined standards facilitated the determination of the composition of the bikunin associated CS (Figure 3b). The analyses showed that the bikunin CS was composed of both unsulfated disaccharides and chondroitin 4-sulfated disaccharides; however, no chondro-di-6-sulfate was detected (Figure 3b). Quantitative analyses of the imaged gels revealed a ratio of 1:3 between sulfated and unsulfated disaccharides.

(b) Length of the CS. Attempts to sequence individual CS chains are complicated by the potential of monosaccharides to display different configurations and linkages (57). To increase the level of confidence in the obtained results, we analyzed the length of the CS by employing three methods

Table 1: Characterization of Lys-C Peptides Derived from Bikunin[a]

HPLC fraction	obsd mass (*m*/*z*)	calcd mass (Da)	sequence
1	672.4	672.74	K7: FYSEK
2	3205.4	2128.32	K1*: AVLPQEEEGS*GGGQLVTEVTK
3	1767.5	1767.07	K6: CVLFPYGGCQGNGNK
4	2094.0	2094.31	K8: ECREYCGVPGDGDEELL
5	2598.7	2598.86	K8: ECREYCGVPGDGDEELLRFSN
6	7973.1	5630.34	K3*: EDSCQLGYSAGPCMGMTSRYFYN*GTSMACETFQYGGCMGNGNNFVTEK
7	2397.2	2397.67	K8: ECREYCGVPGDGDEELLRF
8	4243.5	4247.10	K4#: ECLQTCRTVAACNLPIVRGPCRAFIQLW#AFDAVK
9	4213.6	4215.10	K4: ECLQTCRTVAACNLPIVRGPCRAFIQLWAFDAVK

[a] HPLC-purified peptides were analyzed by MALDI-TOF and Edman degradation. The obtained masses and the putative sequences are shown. Amino acid residues identified by Edman degradation are italicized. Glycosylated (*) and oxidized (#) peptides and residues are labeled as indicated. Two truncated C-terminal variants and the intact C-terminal were identified (peptides K8). In addition, the *N*- and O-linked glycosylated peptides were apparent (peptide K1 and K3). We did not detect other posttranslation modifications of IαI-derived bikunin.

Table 2: Sequential Glycosidase Digestion of the Lys-C Derived Glycopeptide K2[a]

added glycosidase	obsd mass (*m*/*z*)	calcd mass (Da)	removed monosaccharides
	7968.3	7964.52	
neuraminidase	7393.8	7382.00	2 *N*-acetylneuraminic acid
galactosidase	7057.2	7057.32	2 galactose
N-acetylglucosaminidase	6654.2	6651.32	2 *N*-acetylglucosamine
N-glycosidase F	5776.0	5759.50	core region

[a] The masses were determined by MALDI-MS and compared to masses calculated for the glycosylated peptide. (The slightly higher masses observed are most likely caused by partial oxidation of the three methionine residues in this peptide.)

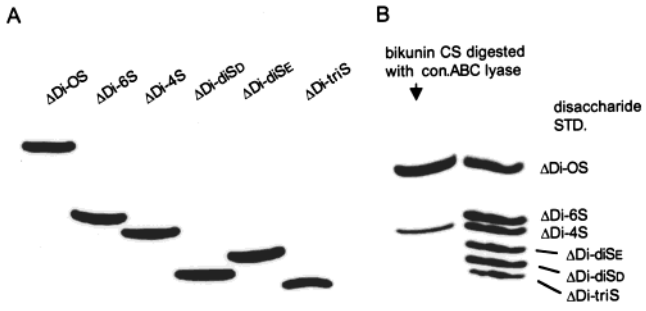

FIGURE 3: Disaccharide composition of the Ser_{10} CS chain. Purified bikunin was digested using chondroitin ABC lyase. Standard unsaturated disaccharides and bikunin derived disaccharides were labeled using 2-aminoacridone and run in acrylamide gel electrophoresis. The standard unsaturated disaccharides were run separately to identify their migration position (panel A). The bikunin derived disaccharides were subsequently analyzed next to a mixture of the standard unsaturated disaccharides (panel B). The analysis showed the presence of both unsulfated disaccharides and chondroitin 4-sulfate disaccharides; however, no chondro-di-6-sulfate was detected. Quantitative analysis of the imaged gel revealed a ratio of 1:3 between sulfated and unsulfated disaccharides.

and two fundamentally distinct protocols. Two of the methods were based on labeling the reducing end of the CS and subsequently estimating the size by acrylamide gel electrophoresis. The other method used MALDI-MS to determine the mass of bikunin. On the basis of the theoretical mass of the polypeptide, along with the mass of other modifications, the length of the CS could be calculated.

Samples were labeled at the reducing terminal xylose of the CS using the uncharged iodinatable compound, *p*-aminophenol, or the fluorophore ANTS, which contributes three negative charges to labeled oligosaccharides. The difference in charge between these compounds is likely to affect the migration of the shorter oligosaccharides. The sizes of the products were estimated by gradient PAGE followed by imaging as appropriate. A partial chondroitin ABC lyase digest of CS resulted in fragments with the general structure ΔHexUA-(GalNac-GlcUA)$_n$-Gal-Gal-Xyl-R, where "R" is ^{125}I *p*-aminophenol or the flororesent label described above. The migration of these fragments was compared to the migration of the intact CS released from bikunin after β-elimination (Figure 4, panels A and B).

The ^{125}I-*p*-aminophenol-labeled CS displayed a marked polydispersity, ranging in size from approximately 5 to 20 disaccharide repeats with an average of $n = 15$ (Figure 4, panel A). The assignments of the CS chain sizes were based on a comparison with fragments obtained after partial degradation with chondroitin ABC lyase (Figure 4, panel A). Because the migration of some saccharides ($n = 1-3$) were anomalous due to low charge density of the region near the reducing end (*52*), their assignments were confirmed by size exclusion chromatography (Bio-Bel P-6) (data not shown). These results indicated that the more distant segments were not sulfated. The use of the ANTS labeling method is likely to compensate for the low charge density of the shorter oligosaccharides because the label contributes three negative charges to the segment. (Figure 4, panel B). The two methods yielded very similar results (Figure 4, panels A and B), and these data suggest that the CS associated with Ser_{10} is composed of 15 ± 3 [GlcUA-GalNAc] disaccharides units.

The length of the CS was also determined by MALDI-MS. The purification of a peptide containing Ser_{10} and the complete CS proved difficult because of the high carbohydrate to peptide ratio. Protocols based on HPLC or ion exchange chromotography were likely to disperse the potentially heterogeneous sample and result in the purification of only a subfraction. To avoid these problems, we analyzed the intact bikunin molecule directly without further purification. These analyses were feasible because we had previously characterized all other modifications, including

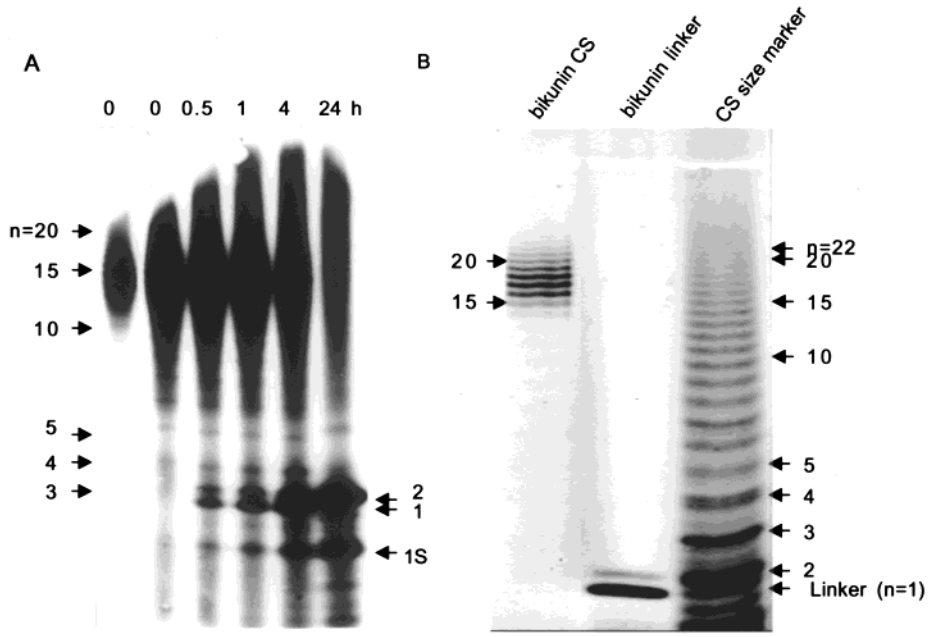

FIGURE 4: Sequence analysis of the bikunin CS chain. The sequence of the CS was investigated with use of two different protocols. In some experiments the bikunin CS was released from the protein core by β-elimination of the $Ser_{10}-Xyl$ bond, and the reducing terminal xylose was labeled with ^{125}I-*p*-aminophenol. The radioactive CS chains were then analyzed directly or after partial chondroitin ABC lyase. The general structure of the observed fragments were ΔHexUA-(GalNAc-GlcUA)$_n$-Gal-Gal-Xyl-^{125}I-*p*-aminophenol, with "*n*" being the number of disaccharide repeats. The recovered and labeled CS chains were subjected to gradient PAGE (panel A). The fragments were also separated by gel chromatography on Bio-Gel P-6 (data not shown), and individual CS fragments were subjected to electrophoresis to confirm their migration positions. The migration positions of CS fragments with $n = 3-20$ are indicated at the left (panel A). Note that the migration of saccharides with $n = 1$, 2, or 3 deviate due to the lack of charges in the linkage region Gal-Gal-Xyl-R. 1S is presumably sulfated variant of saccharide $n = 1$ (panel A). In other experiments the bikunin CS was released from the protein core by β-elimination of the $Xyl-Ser_{10}$ bond. The released CS was labeled with ANTS without further treatment and subjected to gradient PAGE (panel B). The CS size markers in these experiments were generated by limited chondroitin ABC lyase digestion of CS followed by ANTS labeling. The general structure of these fragments was (ΔHexUA-GalNAc)$_n$-ANTS, with *n* being the number of disaccharide repeats. The position of the CS "stub" ΔHexUA-GalNAc-GlcUA-Gal-Gal-Xyl-ANTS was determined by analysis of a completely degraded bikunin CS (Panel B). Note that the CS "stub" displays two bands. These bands are most likely ΔHexUA-GalNAc-GlcUA-GalNAc-GlcUA-Gal-GXyl-ANTS and ΔHexUA-GalNAc-GlcUA-Gal-Gal-Xyl-ANTS. The results of these experiments show that the bikunin CS is heterogeneous in length with ~15 disaccharide repeats.

the homogeneous Asn_{45} N-linked glycan and the truncated C-terminus. With this information, the detected variations in the mass of intact bikunin could then be elucidated and ascribed to the SC.

The molecular mass of intact bikunin (equivalent to the high molecular mass form in SDS−PAGE) was determined to be 24636 Da (Figure 5, panel A). Subsequent complete enzymatic degradation of the CS by chondroitin ABC lyase reduced the mass to 18 858 Da (Figure 5, panel B). The average mass of a disaccharide repeat is 399 Da on the basis of the previously determined ratio of 1:3 between sulfated (459 Da) and unsulfated (379 Da) disaccharides (see Figure 5). The mass difference between intact and chondroitin ABC lyase degraded bikunin (5778 Da), divided by the average mass of a disaccharide (399 Da), is equivalent to 14.5 disaccharide repeats.

Pronounced heterogeneity was apparent in the spectra indicated by the broad peaks (Figure 5, panel A). The peak width measured at half-maximum amplitudes stretches approximately 2500 Da, corresponding to the mass of approximately 6 disaccharide repeats. However, the peak broadening was reduced from 2500 to 1250 Da by chondroitin ABC lyase digestion (Figure 5, panel B). Subsequent analysis following neuraminidase digestion revealed that the residual peak brodening was caused by loss of sialic acids from the N-linked glycan during the analysis (Figure 5, panel C). Two variants at *m*/*z* 18 275 and *m*/*z* 18 764 were detected in this analysis. The mass difference of 489 Da suggests that one disaccharide unit (459 Da) was attached to -Gal-Gal-Xyl- as a result of the decreased affinity of chondroitinase ABC lyase for this disaccharide (Figure 5, panel C). Alternatively, a sulfate group could be attached to the ΔHexUA-GalNac-GlcUA-Gal(SO_4)-Gal-Xyl- as previously suggested (*56*). The additional disaccharide unit attached to the -Gal-Gal-Xyl region was also identified during the FACE sequencing experiments (see Figure 4, panel B, lane "bikunin stub").

We conclude that the peak broadening observed during the analysis of intact bikunin was caused by loss of sialic acids from the N-linked glycan and heterogeneity in the length of the CS. Since the removal of sialic acid only reduced the peak broadening by 1250 Da, the remaining 1250 Da was caused by CS polydispersity. A 1250 Da mass variation divided by the average mass of a disaccharide (399 Da) is equivalent to ± 3 disaccharides. Taken together, the MALDI analysis shows that the bikunin CS consists of 14.5 $\pm$ 3 disaccharide repeats.

(c) Relative Position of HC1 and HC2 on the CS. The order in which the heavy chains of IαI are positioned relative to each other and to bikunin is unknown. To investigate this, we submitted IαI to limited chondroitin ABC lyase digestion (Figure 6). The results showed that whereas only a small amount of enzyme was required to dissociate bikunin, a much higher concentration was needed to dissociate HC1·HC2

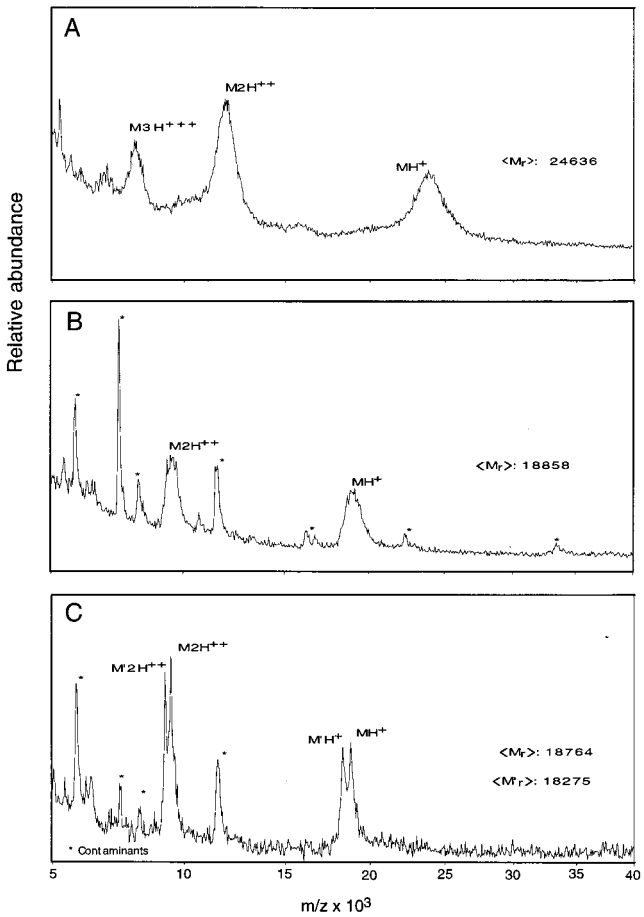

FIGURE 5: Mass determination of intact bikunin by MALDI-MS. The masses of intact bikunin (panel A), bikunin after chondroitin ABC lyase digestion (panel B), and bikunin after a combined chondroitin ABC lyase and neuraminidase digestion (panel C) were determined. The analysis of intact bikunin resulted in very broad peaks caused by a combination of heterogeneity in the length of the CS and metastable loss of sialic acid from the N-linked glycan. The mass difference between intact bikunin and chondroitin ABC lyase digested bikunin was 5778 Da, and the examination of these data and similar experiments suggests that the CS was 14.5 ± 3 disaccharides units long (see text for details).

(Figure 6, panels A and B). The relative ease by which bikunin was released, along with the much more resistant dissociation of the heavy chains, suggests the heavy chains are positioned toward the nonreducing end of the ~15 disaccharide chain and the two heavy chains are positioned relatively close together.

(d) Order of the Two HC1 and HC2 on the CS. It is evident that either HC1 or HC2 is positioned closer to the reducing end of the CS and this arrangement may or may not be conserved in all molecules. The CS was first digested by chondroitin ABC lyase under limited conditions. This procedure released bikunin and degraded most of the CS, but it left the heavy chains attached to a presumably short CS fragment. The reducing end of this truncated CS was labeled using the flourophor ANTS. After removal of excess reagent, HC1·HC2 were dissociated by extensive chondroitin ABC lyase digestion, and the derivatized heavy chains were resolved by reduced SDS−PAGE. The position in the gel was compared to that of HC1 and HC2 that had been derivatized after extensive chondroitin ABC lyase digestion (Figure 7). Moreover, all protein bands were transferred to PVDF membranes and identified by Edman degradation (not

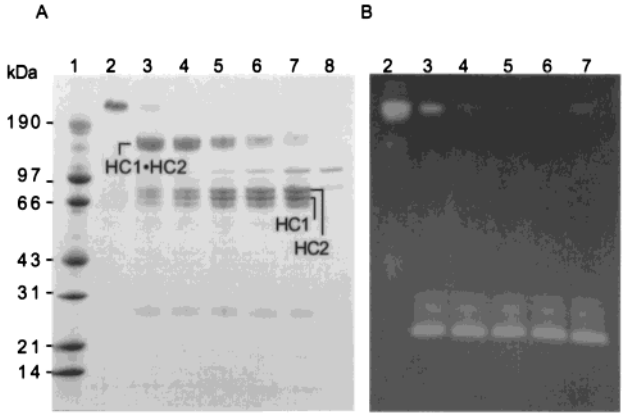

FIGURE 6: HC1 and HC2 positioned in close proximity on the CS. Although the molecular structure of the cross-link is known (*30*), the overall arrangement of HC1 and HC2 in relation to bikunin is not. To answer this question of arrangement IαI was titrated with chondroitin ABC lyase (panels A and B, lanes 2−7), and aliquots were analyzed by SDS−PAGE. Chondroitin ABC lyase is shown alone for comparison (panel A, lane 8). The gels were stained with Coomassie blue (panel A) and for trypsin inhibitory activity using the trypsin inhibition counter-staining technique (*12*, *40*) (panel B). The latter staining method specifically detects bikunin. A relatively low concentration of enzyme released bikunin (panels A and B, lane 3), but significantly higher concentrations were required to hydrolyze the *N*-acetylgalactosamine linkages between the heavy chains to dissociate HC1·HC2 (panel A, lanes 6 and 7). These results suggest that HC1 and HC2 are positioned in close proximity and that the distance from bikunin to the heavy chains is significantly greater than the distance between the heavy chains.

shown). Examination of the gel showed that only HC2 was labeled by ANTS suggesting that HC2 is positioned toward the reducing end of the CS, and HC1 is positioned toward the nonreducing end. The experiments suggest the following organization of the heavy chains in relation to the CS: $[GlcUA\text{-}GalNAc]_a$-HC1-$[GlcUA\text{-}GalNAc]_b$-HC2-$[GlcUA\text{-}GalNAc]_c$-Gal-Gal-Xyl-Ser_{10}-bikunin ($a + b + c = 12-18$ disaccharides). The relative size of "*a*" is not known, but the data suggest that "*b*" is significantly smaller than "*c*".

Purification and Analysis of Bikunin Isomers. To analyze the heterogeneity of the CS, we separated intact bikunin by anion exchange chromatography at pH 3.4. At this pH, negative charges of the molecule are confined to the sulfate groups of the CS. The analysis resulted in four peaks (Figure 8). The corresponding fractions were analyzed by MALDI-MS. These spectra displayed broad peaks similar to bikunin before anion exchange chromatography (see Figure 5, panel A), indicating that significant heterogeneity is present within each variant. The measured average mass of each isoform differed by approximately 80 Da (Figure 8). The overall mass difference observed between the four isotypes was approximately 260 Da, significantly below the mass of a single condro-disaccharide unit (379 Da). Hence, the determined masses strongly indicate that the differences between the bikunin isotypes are caused by the number of sulfate groups present (80 Da/sulfate group).

DISCUSSION

This study was designed to examine the structure of the CS and the overall organization of the IαI subunits in relation to the CS. We have previously shown that IαI is composed of one bikunin molecule and two heavy chains, HC1 and

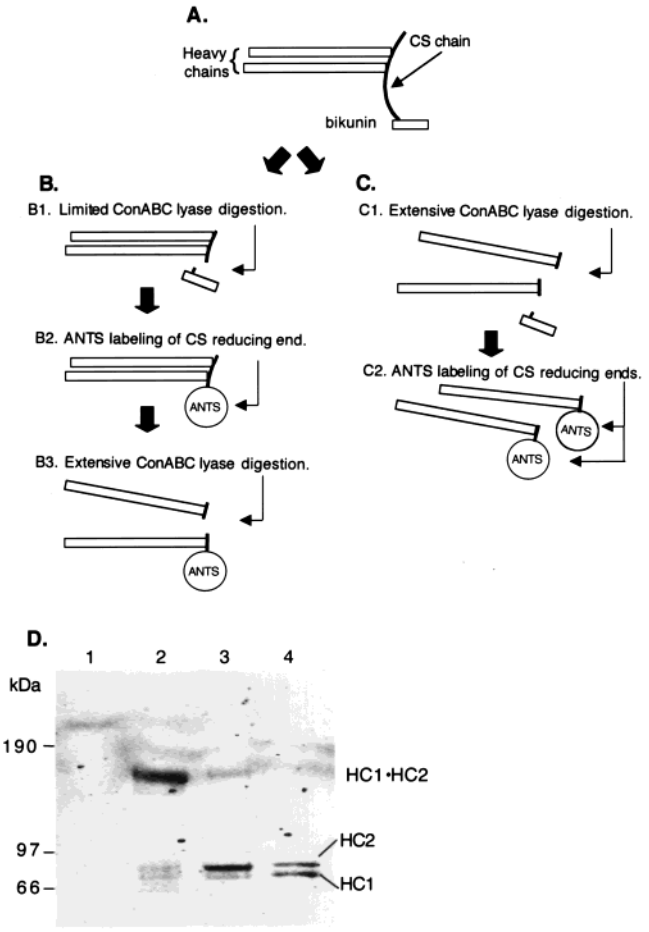

FIGURE 7: Organization of the HC1 and HC2 on the CS chain. It is evident that either HC1 or HC2 of IαI is positioned closer to the reducing end of the CS chain (see schematic in panel A). To examine this question of order, IαI was digested with chondroitin ABC lyase to release bikunin and most of the CS (panel B1). The conditions were optimized to remove the CS without dissociating HC1·HC2. The HC1·HC2 complex was then derivatized with ANTS to label the reducing end of the CS (panel B2). This complex was digested extensively with chondroitin ABC lyase to dissociate the HC1·HC2 complex (panel B3). To identify the position in SDS−PAGE of the dissociated heavy chains, IαI was digested extensively with chondroitin ABC lyase (panel C1) and then labeled with an ANTS (panel C2). The products of these analyses were resolved by reduced SDS−PAGE (panel D): IαI before chondroitin ABC lyase digestion but after ANTS derivatization (lane 1; see panel A); IαI after limited chondroitin ABC lyase digestion and ANTS derivatization (lane 2; see panel B2); HC1·HC2 after ANTS derivatization and extensive chondroitin ABC lyase (lane 3; see panel B3); dissociated HC1 and HC2 followed by ANTS derivatization (lane 4; see panel C2). The acquired image was analyzed using the FACE software densitometer (version 2.3). All bands were identified by Edman degradation after transfer to PVDF membranes. The results show that the top band, which corresponded to HC2, was labeled with ANTS. This show that HC2 is positioned toward the reducing end and HC1 is positioned away from bikunin toward the nonreducing end of the CS.

HC2 (*12*). The intercellular assembly of IαI (*28*) involves a novel covalent "PGP" cross-link formed between the C-terminal Asp residues of the heavy chains and the CS (*30*). In the original studies, the molecular structure of the PGP cross-link was determined after fragmentation of the CS (*30*, *31*). Consequently, questions about the gross architecture of the interaction remain: specifically, (i) what is the length of the CS, (ii) what is the order of the heavy chains on the CS relative to bikunin, (iii) is this arrangement conserved in

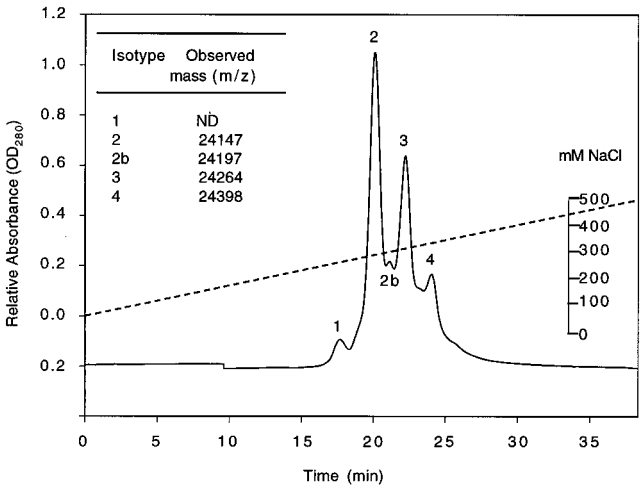

FIGURE 8: Separation of bikunin charge isoforms. Purified bikunin was analyzed by anion exchange chromatography at pH 3.4. The NaCl gradient is indicated (---). The negative charge of the molecule at this pH can be ascribed mainly to the sulfate groups on the CS. The analysis resolved four isoforms with successively larger masses (see insert). The roughly 80 Da increase in mass suggests each isoform deviated by one sulfate group. The mass spectra displayed significant peak broadening (similar to the spectra in Figure 5, panel A), a finding that suggests the heterogeneity in length was maintained in the isoforms. The spectra were internally calibrated by addition of apomyoglobin.

all IαI molecules, (iv) are the attachment sites conserved in all IαI molecules, and if so (v) which specific disaccharide units carry the heavy chains? To begin to address these questions, we first mapped the bikunin posttranslational modifications not associated with Ser_{10}.

IαI-derived bikunin was purified, and the Ser_{10}-associated CS chain was removed by limited SPaseV8 digestion. The analyses of the truncated bikunin molecule indicated that the predicted N-glycosylation site Asn_{45} was fully utilized, as has previously been observed in urinary trypsin inhibitor (*55*). Further analysis of the Asn_{45} glycan verified the presence of a homogeneous biantennary complex glycan composed of 5 hexose and 4 *N*-acetylhexosamine monosaccharides capped with two sialic acid residues (Figure 9). Some glycan heterogeneity is usually observed, but this N-linked glycan appeared to be homogeneous. In addition, we observed C-terminal processing at Leu_{143}-Arg and Phe_{145}-Ser during MALDI-MS analyses of the N-terminally truncated bikunin molecule. Both the processed and unprocessed C-terminals were identified, and the ratio of processed to intact C-terminal was determined to be approximately 3:1. The functional significance, if any, of this observation is unclear, but the C-terminus of bikunin appears to be exposed (*58*) and could be susceptible to proteolytic "trimming" during purification.

The analyses of the Ser_{10} CS suggested that it is composed of ∼15 disaccharide repeats. Some polydispersity of the CS was observed, and this variation in length is not the result of fragmentation during purification since it remained consistent even after extensive handling of the CS and between different preparations. The degree of sulfation also varied; on average, every fourth disaccharide was sulfated. To further examine the CS sulfation heterogeneity, we analyzed bikunin by analytical anion exchange chromatography at pH 3.4. At this pH, the interactions between bikunin and the column depend primarily on the negative charges associated with the sulfate groups. The four isostypes

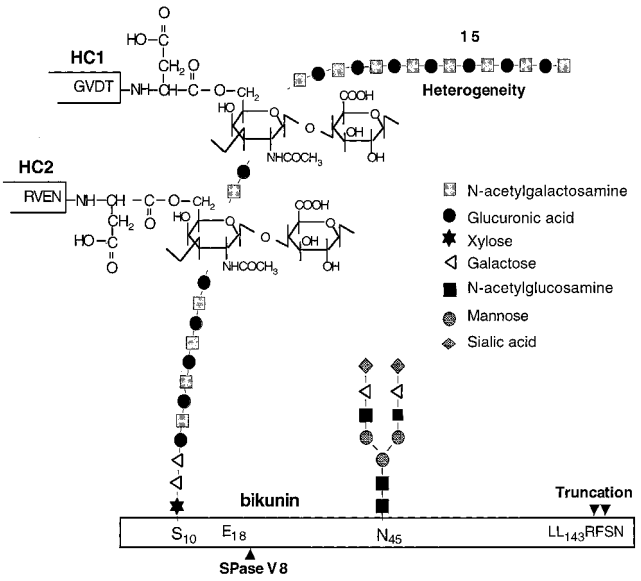

FIGURE 9: Schematic summarizes the results obtained in this study. Bikunin contained three posttranslational modifications: (i) glycosylation of Ser_{10}, (ii) glycosylation of Asn_{45}, and (iii) truncation of the C-terminal. We did not detect any other posttranslational modifications. The Ser_{10} CS was composed of 15 ± 3 [GlcUA-GalNAc] disaccharide units. The ratio between sulfated and unsulfated was 1:3, and most of the sulfations appeared to be near the reducing end. The structure of the Asn_{45} associated glycan was shown to be a homogenous "complex type" biantennary structure as illustrated. Approximately 25% of the bikunin molecules were truncated at the $Leu_{143}-Arg$ peptide bond, 12% were cleaved at the $Phe_{145}-Ser$ peptide bond, and the remaining molecules were full length. HC2 is positioned closer to bikunin than is HC1, and both heavy chains are positioned toward the nonreducing end, possibly on adjacent disaccharide units, leaving a long "stalk" between the heavy chains and bikunin. The structure of the PGP cross-link is indicated.

resolved deviated from each other by the mass of approximately one sulfate group. The polydispersity was present in each isotype, suggesting that the sulfated disaccharides were more prevalent near the reducing end and not in the heterogeneous end of the CS (nonreducing). This notion was supported by experiments in which the reducing terminal xylose of the CS was labeled using the uncharged *p*-aminophenol. These labeling experiments were performed on CS purified from a combination of the four isoforms. PAGE analysis of these *p*-aminophenol-labeled saccharides ($n = 1-3$) revealed an abnormal migration due to a low charge density of the linkage region. Other more highly charged, and therefore probably sulfated, variants of $n = 1$ and $n = 2$ were also seen. These are likely the sulfated saccharides responsible for the bikunin charge heterogeneity observed at pH 3.4. Consequently, the observed sulfate heterogeneity present in the four isoforms seems to be associated mainly with the region near the reducing end. Overall the Ser_{10}-associated CS was heterogeneous in length and in the degree of sulfation.

The order in which IαI heavy chains are attached to the CS was analyzed by fragmenting the CS and identifying the protein components that remained associated on the CS. Limited enzymatic degradation of the CS resulted in the immediate release of bikunin. Since only catalytic amounts of CS degrading enzyme were required to dissociate bikunin, it is likely that the heavy chains are positioned on a long "stalk" toward the nonreducing end. Much higher concentrations of enzyme were required to dissociate the two heavy chains; this finding suggests that the heavy chains are relatively close and may be on adjacent GalNAc units. To determine which heavy chain was positioned toward the reducing end of the CS, conditions were identified that did not dissociate the heavy chains but did degrade the CS not linking the heavy chains. The reducing end of this degraded CS was then labeled before the CS was cleaved between the heavy chains. This procedure allowed the identification of the heavy chain that carried the reducing end of the CS and thus was positioned closest to bikunin. The results show that HC2 was positioned toward the reducing end of the CS, HC1 was positioned toward the nonreducing end, and this arrangement was conserved in all IαI molecules (Figure 9).

IαI is synthesized by hepatocytes and is assembled intercellularly (*28*). The details of the assembly mechanism are still rudimentary, but the data presented here suggest that the mechanism is specific and selective since the $[GlcUA-GalNAc]_a$-HC1-$[GlcUA-GalNAc]_b$-HC2-$[GlcUA-GalNAc]_c$-Gal-Gal-Xyl-Ser_{10}-bikunin arrangement is conserved in all IαI molecules. Moreover, HC3 of pre-α-inhibitor has not been found in association with other heavy chains and is not selected during the biosynthesis to form trimers. Interestingly, the sulfated disaccharides of the CS appeared to be concentrated near bikunin away from the attached heavy chains. The associated negative charges might play a role during the biosynthesis and assembly of IαI to guide the positioning of the heavy chains.

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