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The interaction of enoxaparin and fondaparinux with calcium

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ABSTRACT

The main sites of calcium binding were determined for the low molecular weight heparin drug enoxaparin and the synthetic pentasaccharide Arixtra (fondaparinux). [^{1}H,^{13}C] HSQC pH titrations were carried out to characterize the acid–base properties of these samples both in the presence and absence of calcium. The differences in the titration curves were used to determine the structural components of enoxaparin and fondaparinux responsible for Ca^{2+} binding. In enoxaparin both unsubstituted and 2-*O*-sulfated iduronic acid residues are important in calcium binding and the presence of the 2-*O*-sulfo group does not seem to influence the Ca^{2+} binding capability of the iduronate ring. In fondaparinux changes in chemical shifts upon Ca^{2+} binding were smaller than observed for enoxaparin, and were observed for both the glucuronic acid and 2-*O*-sulfated iduronic acid residues. In enoxaparin significant perturbations of the chemical shift of the *N*-sulfoglucosamine anomeric carbon in residues connected to 2-*O*-sulfated iduronic acid were detected on Ca^{2+} binding, however it was not possible to determine whether these changes reflect direct involvement in calcium complexation or result from through space interactions or conformational changes.

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1. Introduction

The molecular-level characterization of glycosaminoglycans (GAGs) is of critical importance to advancing our understanding of the relationships between elements of structure and the biological processes mediated by these biopolymers.[1] GAGs are a class of linear, usually highly anionic polysaccharides composed of repeating hexuronic acid–hexosamine disaccharide units. In heparin the hexuronic acid component is either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA), which may be 2-*O*-sulfated. The hexosamine residue is D-glucosamine (GlcN), which may be sulfated at the 3-*O* and 6-*O* positions and *N*-sulfated (GlcNS), N-acetylated (GlcNAc) or present as an unsubstituted primary amine.[2] In heparin, these monosaccharide building blocks are connected through (1→4) linkages.

Heparin is a widely used anticoagulant drug prescribed as unfractionated heparin, low molecular weight heparin (LMWH) and as a synthetic heparin mimetic. LMWHs are prepared by chemical or enzymatic depolymerization reactions that produce complex and polydisperse mixtures of heparin-derived oligosaccharides that may be structurally modified at the non-reducing and/or reducing ends as a result of the process used for their preparation.[3] The LMWH drug enoxaparin is administered as a subcutaneous injection to prevent and treat deep vein thrombosis or pulmonary embolism.[4] It is a heterogeneous mixture of oligosaccharides with an average molecular weight of 4.4 kDa. It is prepared through reaction of unfractionated heparin to form benzyl esters with the uronic acid carboxylate groups followed by base treatment to facilitate β-elimination.[5] In addition to reducing the average size of the heparin chains, the depolymerization reaction produces structural modifications to the resulting oligosaccharides including introduction of a double bond to form a 4–5 unsaturated uronic acid (ΔUA) residue at the non-reducing end of the cleaved chain that is mainly 2-*O*-sulfated (ΔUA2S).[6] Chemical β-elimination can also generate bicyclic 1,6-anhydrosugar side-products introducing additional heterogeneity into the enoxaparin preparation (Fig. 1A). Monosaccharide analysis of enoxaparin reported that 4.4% of the aminosugars contained the 1,6-anhydro structure.[7] The synthetic pentasaccharide fondaparinux sodium (Arixtra™) (Fig. 1B) was designed to mimic the heparin pentasaccharide responsible for high affinity antithrombin-III binding and accounts for a large fraction of the synthetic heparin market.[8]

In addition to its anticoagulant activity, heparin interacts with a number of extracellular proteins, including fibroblast growth factors, to modulate their activity.[9,10] The unique conformational flexibility of IdoA serves to facilitate the approach of the anionic groups of the GAG to appropriate basic groups of a protein ligand.[11] Several of these heparin–protein interactions involve cations.[12–14] For example, the heparin binding of the anticoagulant, calcium- and membrane-binding protein Annexin V is calcium-dependent.[12] Calcium also inhibits the heparin acceleration of the anticoagulant serpin, a protein Z-dependent protease inhibitor, by reducing its affinity for heparin.[14]

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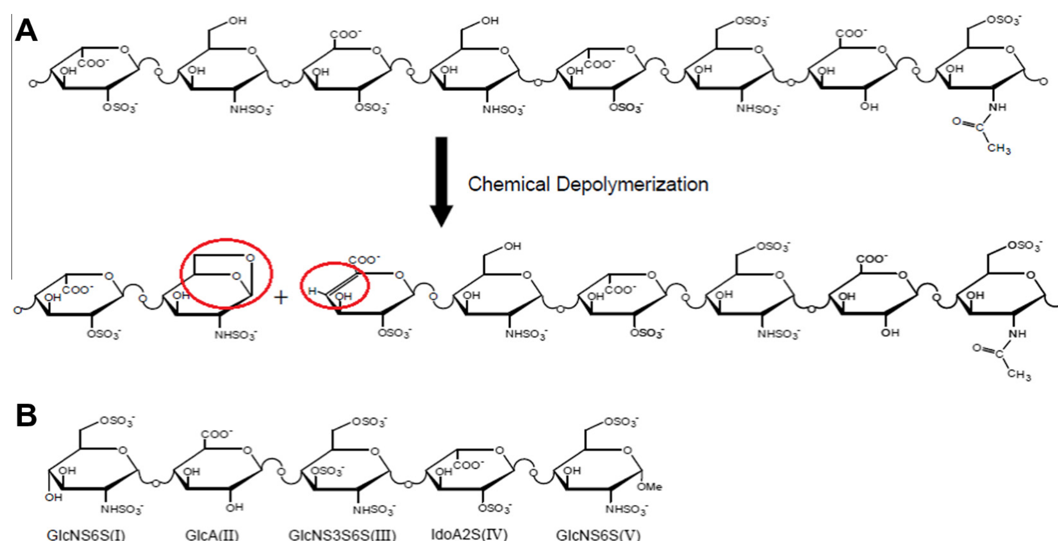

Figure 1. (A) Example products produced by the chemical depolymerization of unfractionated heparin to form enoxaparin. (B) Structure of the synthetic fondaparinux pentasaccharide.

Previous NMR studies indicate that Na^{+}, Ca^{2+}, and Mg^{2+} interact at low pH with the protonated carboxylic acid form of heparin through delocalized, long-range electrostatic interactions. At higher pH values, however, there is a site-specific contribution to the binding of Ca^{2+}.[15]

Because of its physiological relevance, the structural nature of the interaction between Ca^{2+} and heparin has been previously investigated by ^{1}H NMR spectroscopy and molecular dynamics calculations with the aid of model hexasaccharides.[15–17] The acid–base chemistry of the carboxylic acid groups of heparin and heparin-derived oligosaccharides has previously been studied using ^{13}C NMR[18] as well as through the measurement of ^{1}H NMR spectra as a function of pD.[16,19] NMR spectral changes of the IdoA residue reflect the involvement of the carboxylate moiety in calcium binding. Calcium ion complexation shifts the titration curve of the heparin IdoA-H5 proton to lower pD, corresponding to an apparent increase in the acidity of the carboxylic acid group, and the largest displacements of NMR chemical shifts on addition of Ca^{2+} were observed for the IdoA-H1 and H5 resonances.[15] The carboxylate groups of the IdoA residues are essential for Ca^{2+} specific binding as this capability is lost when heparin is chemically modified as the methyl ester or when these groups are protonated.[16] A similar role in Ca^{2+} binding has been attributed to the sulfamido group of the glucosamine residues as heparin fragments lacking of this function are unable to bind Ca^{2+} as in unmodified heparin.[20,21] The results of the titration of hexasaccharides with Ca^{2+} suggested that the 6-*O*-sulfate group of the glucosamine ring was also essential for the interaction with Ca^{2+} while the 2-iduronate substituent appeared to be redundant.[17] However, based on molecular dynamics calculations the main sites of Ca^{2+} binding were suggested to be the IdoA carboxylate and 2-*O*-sulfate groups, and the GlcNS sulfamate ($NHSO_3^-$) moiety.[15]

The aim of the current study was to investigate the main sites of Ca^{2+} binding in enoxaparin and fondaparinux, and to clarify the controversial roles the IdoA 2-*O*-sulfate and GlcNS *N*-sulfate groups play in the complexation process.

2. Experimental

2.1. Materials

Enoxaparin sodium was obtained from the US Pharmacopoeia (Rockville, MD), with an average molar mass of 4421 Da. Sodium chloride was purchased from Fisher Scientific (Pittsburgh, PA), while 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt (DSS) and calcium acetate were obtained from the Sigma Chemical Company (St. Louis, MO). Deuterium oxide, DCl, and NaOD were purchased from Cambridge Isotope Laboratories (Andover, MA). Fondaparinux sodium, sold as Arixtra in the United States, was obtained through the University Pharmacy and Department of Pharmacy Administration of Semmelweis University, formulated as prefilled syringes. The fondaparinux sodium solutions were pooled and desalted using a 1.6 × 70 cm Sephadex G10 superfine column (GE Healthcare, Pittsburgh, PA) with a flow rate of 0.15 mL/min using high-performance liquid chromatography (HPLC) grade water (Burdick & Jackson, Honeywell, Morristown, NJ) as the eluent.

2.2. Sample preparation for the NMR pH titrations

A 12.3 mg/mL enoxaparin sodium stock solution was prepared in D_2O containing 0.15 M NaCl. For the titration in the presence of calcium, a 22.8 mg/mL enoxaparin sodium stock solution was prepared containing 0.15 M NaCl and 69.3 mM calcium acetate. A 1.00 mM fondaparinux solution containing 0.15 M NaCl was used for pH titrations in the absence and the presence of 5 mM calcium acetate. All [^{1}H,^{13}C] HSQC chemical shift titrations were carried out using 600 μL samples in 5 mm NMR tubes over the pD range 1–8, with spectra measured at 10–12 different pD values per titration.

An Orion 9110DJWP double junction pH electrode was calibrated with standard buffer solutions at 2.00, 4.00, 7.00, and 10.00 (Fisher Scientific). The pH meter readings for D_2O solutions were converted to pD using the equation $\text{pD} = \text{pH}_{\text{meter reading}} + 0.4$.[22] The sample pD was adjusted using concentrated DCl and NaOD diluted with D_2O. Chemical shifts measured as a function of pD were subjected to non-linear parameter fitting using Eq. 1 with Origin 8 software (OriginLab, Northampton, MA)

$$\delta^{\text{obsd}} = \frac{\delta_{\text{COO}^-} + \delta_{\text{COOD}} 10^{\text{p}K_a - \text{pD}}}{1 + 10^{\text{p}K_a - \text{pD}}} \quad (1)$$

where δ^{obsd} is the measured chemical shift at a given pD, and δ_{COO^-} and δ_{COOD} are the limiting chemical shifts of the basic and acidic species, respectively. The pK_a values measured in D_2O were converted to aqueous pK_a values using the equation: pK_a (H_2O) = pK_a (D_2O) − 0.6.[18]

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