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Susceptibility of enoxaparin reducing end amino sugars to periodate oxidation

Anna Alekseeva *, Stefano Elli, Cesare Cosentino, Giangiacomo Torri, Annamaria Naggi

Istituto di Ricerche Chimiche e Biochimiche 'G. Ronzoni', via G. Colombo 81, 20133 Milan, Italy

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ABSTRACT

There is a growing interest on glycol-split low-molecular weight heparins (gs-LMWHs), obtained by periodate oxidation of LMWHs, optionally followed by borohydride reduction, as potential anticancer and anti-inflammatory drugs. However, their structural characterization is still a challenging task, mainly because of the high microheterogeneity of the starting material. In addition, susceptibility to oxidation of some end-groups of LMWHs induces additional heterogeneity, making analysis of gs-LMWHs more complex. In our previous study we showed that 1,6-anhydro-D-mannosamine *N*-sulfate was affected by periodate, while its epimer 1,6-anhydro-D-glucosamine *N*-sulfate was resistant. In order to understand the apparently anomalous behavior of terminal 1,6-anhydro-D-mannosamine *N*-sulfate residues, in the present work we have studied by NMR spectroscopy and LC/MS the behavior of the reducing end amino sugar residues of the tetrasaccharides, isolated from the LMWH enoxaparin, in the presence of periodate. Their molecular mechanics conformational characterization has been also performed. We have shown that the C(2)–C(3) bond of the 1,6-anhydro-D-mannosamine residue can be split by periodate despite the N-substitution. Moreover, we have found that both terminal D-mannosamine *N*-sulfate and D-glucosamine *N*-sulfate, lacking the 1,6-anhydro-bridge, can be also oxidized by periodate but with a significantly lower rate. The present results suggest that the *cis*-*e*-/*a*-position of OH and $NHSO_3^-$ groups of N-sulfated 1,6-anhydro-D-mannosamine is not the only factor that makes these end residues susceptible to the oxidation. The 1,6-anhydro-bridge that 'blocks' the ring conformation appears another crucial factor for oxidation to occur. Moreover, we have shown that controlling the reaction time could permit to selectively split non-sulfated iduronic acids of enoxaparin chains without oxidizing terminal amino sugar residues, a finding that may be useful to obtain more structurally homogeneous gs-LMHWs.

1. Introduction

Heparin belongs to the glycosaminoglycan family and it is constituted of repeating disaccharide building blocks containing 1,4-linked uronic acid (L-iduronic or D-glucuronic) and D-glucosamine. Regular 'fully sulfated' regions, containing 2-*O*-sulfated iduronic acid (I_{2S}) and *N*-sulfate-glucosamine-6-*O*-sulfate (A_{NS6S}) are prevalent within heparin chains, but are interspersed by undersulfated sequences bearing non-sulfated iduronic (I) or glucuronic (G) acids and N-acetylated glucosamine residues (A_{NAc}). The specific pentasaccharide sequence *N*-acetyl-D-glucosamine-6-*O*-sulfate-α-(1→4)-D-glucuronic acid-β-(1→4)-D-glucosamine *N*,3-*O*,6-*O*-trisulfate-α(1→4)-L-iduronic acid 2-*O*-sulfate-α(1→4)-D-glucosamine *N*,6-*O*-disulfate (A_{NAc6S}-G-A_{NS3S6S}-I_{2S}-A_{NS6S}), present only in some of the chains, constitutes the antithrombin-binding region (ATBR), essential for a high anticoagulant and antithrombotic activity of heparins.[1,2]

Glycol-split (gs) derivatives of heparins with potential application in anti-cancer and anti-inflammation therapies[3,4] are usually obtained by oxidation of vicinal hydroxyl groups of non-sulfated uronic acid residues followed by a reduction step in order to

Abbreviations: gs-LMWHs, glycol-split low-molecular weight heparins; I, L-iduronic acid; G, D-glucuronic acid; I_{2S}, L-iduronic acid 2-*O*-sulfate; A_{NS6S}, D-glucosamine-*N*,6-*O*-disulfate; A_{NAc}, N-acetylated D-glucosamine residues; A_{NS3S6S}, D-glucosamine *N*,3-*O*,6-*O*-trisulfate; M_{NS6S}, D-mannosamine-*N*,6-*O*-disulfate; $1,6aA_{NS}$, 1,6-anhydro-D-glucosamine *N*-sulfate; $1,6aM_{NS}$, 1,6-anhydro-D-mannosamine *N*-sulfate; ΔU_{2S}, 4,5-unsaturated uronic acid 2-*O*-sulfate; R_{am}, remnant of the oxidized terminal amino sugar; ATBR, antithrombin-binding region; RE, reducing end; NRE, non-reducing end; gs1,6a, split 1,6-anhydro-amino sugar; NMR, nuclear magnetic resonance; 2D HSQC, bidimensional heteronuclear single quantum coherence spectroscopy; TOCSY, total correlation spectroscopy; SEC, size-exclusion chromatography; IPRP-HPLC, ion-pair reversed phase high performance liquid chromatography; ESI-Q-TOF-MS, electrospray ionization-quadrupole-time-of-flight mass spectrometer; RT, retention time.

* Corresponding author. Tel.: +39 0270641631; fax: +39 0270641634.
E-mail address: anna.v.alekseeva@gmail.com (A. Alekseeva).

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stabilize the generated aldehydes.[5] The new medical uses of gs-heparins are favored by the loss of anticoagulant activity associated with glycol-splitting of the D-glucuronic acid residue within the ATBR sequence, which is essential for strong binding to, and activation of, antithrombin (AT).[6,7] Other biological activities of heparins, which are not critically dependent on the intact AT-binding sequence, usually are not significantly impaired by glycol-splitting. Moreover, given the increased local mobility induced by gs residues, the flexibility of gs-heparin chains is higher than for unmodified heparins, facilitating in some cases their interactions with proteins.[8,9] Since low-molecular weight heparins (LMWHs) are considered to be pharmacologically more attractive than unfractionated heparins, due to their better bioavailability, also several gs-LMWHs are currently being developed.[10,11]

In spite of such a growing interest on gs-LMWHs, the structural characterization of these non-anticoagulant experimental drugs is still a challenging task, firstly, because of the high microheterogeneity of the starting material (varying also for different origins and manufacture processes). When LMWHs are used as starting materials, the end-groups generated during the depolymerization processes induce additional heterogeneity, making the analysis even more complex.[12]

In our previous work we found that not only the internal non-sulfated uronic acids, but also several terminal residues characteristic for LMWHs are susceptible to periodate oxidation.[12] The most interesting and unexpected behavior was observed for terminal enoxaparin residues. Enoxaparin oligosaccharides bear various amino sugars at their reducing end (RE): glucosamine *N*-sulfate ($A_{NS(6S)}$, ~9% of total amino sugar content[13]) and unnatural residues formed during depolymerization of heparin under alkaline conditions[14], namely mannosamine *N*-sulfate ($M_{NS(6S)}$), 1,6-anhydro-glucosamine *N*-sulfate ($1,6aA_{NS}$), and 1,6-anhydro-mannosamine *N*-sulfate ($1,6aM_{NS}$), about 3%, 2%, and 2.5% of total amino sugar content[13]. It was shown that unlike $1,6aA_{NS}$ residues, the NMR cross peaks of its epimer $1,6aM_{NS}$[13,15] were found to disappear in the HSQC spectrum of enoxaparin upon glycol-splitting.[12] Interestingly, LC/MS analysis of both intact gs-enoxaparin and its heparinase-digest indicated the generation of unknown structures by glycol-splitting. Based on the LC/MS and NMR data, we hypothesized that the splitting of the C(2)–C(3) bond bearing a N-sulfated amino group at the C(2) and a hydroxyl group at the C(3) of $1,6aM_{NS}$ could generate the structures shown in Figure 1.[12] The oxidation by periodate of amino sugars having vicinal OH- and NH_2-groups is a well-known process leading to the splitting of the corresponding C–C bond.[16] However, the presence of different N-substituents induces a variety of oxidative behavior.[16,17] For example, *N*-alkyl-amino alcohols are also susceptible to periodate oxidation but the reaction rate largely varies for secondary and tertiary amines.[16] However, when amino-group is N-sulfated the nucleophilic properties of nitrogen are expected to be diminished, so that periodate oxidation of N-sulfated hexosamines appears unexpected.

The present study is focused on the behavior of terminal amino sugar residues of enoxaparin tetrasaccharides upon periodate oxidation/borohydride reduction. In order to directly prove that the unknown structures found in gs-enoxaparin by LC/MS analysis in our previous work[12] were generated by periodate oxidation of $1,6aM_{NS}$, we isolated from enoxaparin a fraction containing the two isomeric pentasulfated tetrasaccharides ΔU_{2S}-A_{NS6S}-I_{2S}-$1,6aA_{NS}$ and ΔU_{2S}-A_{NS6S}-I_{2S}-$1,6aM_{NS}$ and studied their behavior under glycol-splitting conditions by NMR spectroscopy and LC/MS. To verify the role of the 1,6-anhydro-bridge in the susceptibility toward oxidation a fraction containing the hexasulfated tetrasaccharides ΔU_{2S}-A_{NS6S}-I_{2S}-A_{NS6S} and ΔU_{2S}-A_{NS6S}-I_{2S}-M_{NS6S} have been also studied in the presence of periodate. Molecular mechanics characterization of all target tetrasaccharides has permitted to determine their average conformation in solution, and indicated how their structural differences can influence their behavior toward periodate oxidation.

2. Results

2.1. NMR and LC/MS study of susceptibility of enoxaparin 1,6-anhydro-amino sugars to periodate oxidation

Model compounds were needed to verify whether periodate can split terminal $1,6aM_{NS}$ residues of some enoxaparin oligosaccharides. Since no oligosaccharide standards containing 1,6-anhydro-sugars are available, we performed a multi-step fractionation of enoxaparin components to isolate a fraction containing two isomeric pentasulfated tetrasaccharides ΔU_{2S}-A_{NS6S}-I_{2S}-$1,6aA_{NS}$ and ΔU_{2S}-A_{NS6S}-I_{2S}-$1,6aM_{NS}$ (Scheme 1). The tetrasaccharide fraction was chosen because its structural characterization is easier with respect to longer-chain oligosaccharides. As the first step, SEC-fractionation of the enoxaparin sample was carried out to obtain a fraction enriched in tetrasaccharides, which was, then, sub-fractionated by ion-pair reversed phase high-performance liquid chromatography (IPRP-HPLC) to isolate the target tetrasaccharides (Scheme 1). The obtained fraction was fully characterized by 2D NMR and LC/MS analyses. The corresponding spectral data are shown in Figure S1.

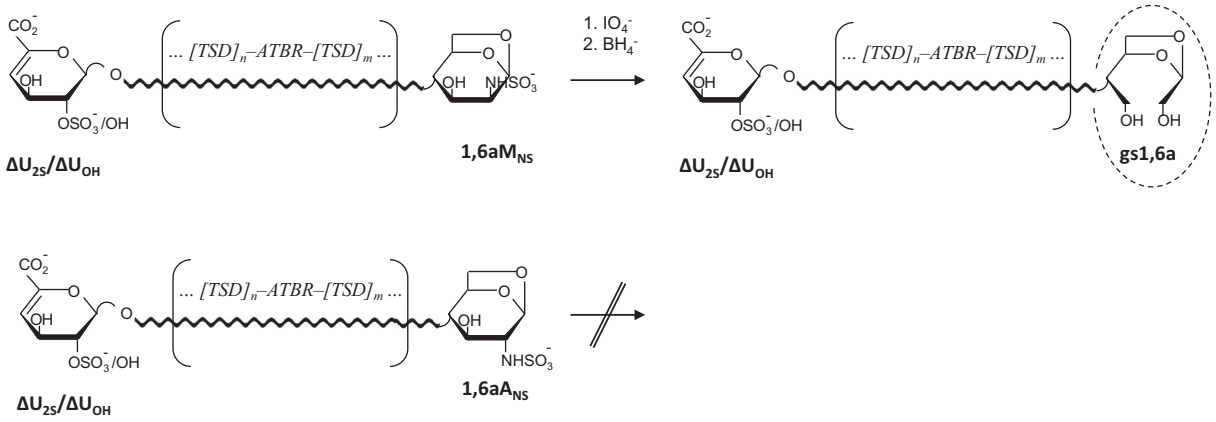

Figure 1. Behavior of N-sulfated 1,6-anhydro-D-mannosamine ($1,6aM_{NS}$) and 1,6-anhydro-D-glucosamine ($1,6aA_{NS}$) residues upon periodate oxidation/borohydride reduction, as previously hypothesized[12] ΔU_{2S} and ΔU_{OH}—2-O-sulfated and non-sulfated 4,5-unsaturated uronic acids, gs1,6a—glycol-split-1,6-anhydro-sugar, TSD—trisulfated disaccharide (I_{2S}-A_{NS6S}) prevalently present within heparin chains.