Gas-Phase and Solution Conformations of Selected Dimeric Structural Units of Heparin

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The molecular structure of four dimeric units (D−E, E−F, F−G, and G−H) of the DEFGH structural unit of heparin, their anionic forms, and their sodium salts have been studied using the B3LYP/6-31+G(d) method. The optimized geometries indicate that the most stable structure of these dimeric units in neutral state is stabilized via "bifurcated" sodium bonds. The equilibrium structure of the biologically active anionic forms of the glycosaminoglycans studied changed considerably in a water solution. The stable-energy conformations around glysosidic bonds found for the individual dimeric species investigated are in agreement with the available experimental structural data for the structurally related heparin-derived oligosaccharides.

1. INTRODUCTION

Heparin (HP) and structurally related heparan sulfate (HS) are a family of macromolecules (glycosaminoglycans) found in virtually all tissues in a wide variety of species.[1−4] HP and HS are chemically similar except for the content of *N*- and *O*-sulfo groups, the content of *N*-acetyl groups, and the ratio of the type of hexuronic acids.[3,5] Sulfo groups in HP appear to play an important role in various biological effects of this polymer.[5,6] Heparin is used in clinics for the prevention and treatment of thrombosis.[2] Its main antithrombotic activity is explained by its ability to potentiate the activity of serine protease inhibitor antithrombin III (AT-III), which inactivates a number of serine proteases—such as thrombin and factor Xa—in the coagulation cascade.[1,6] The action of anticoagulants starts when they bind to antithrombin through a group of five subunits (DEFGH). It was found that this unique pentasaccharide fragment (PS) constitutes the minimal binding domain for AT-III. It is now possible to synthetically produce the active subunit (DEFGH) of heparin,[7−9] which has been recently introduced on the market (fondaparinux sodium).[10] The preparation of PS and its many analogues led to the establishment of the structure−activity relationships of heparin-like pentasaccharides.[11] Despite its importance, the molecular structure of heparin is not known. We know the chemical structure of the disaccharides that might be in a heparin chain.[12] Despite these interesting properties, the glycosaminoglycans remain one of the structurally less-well characterized classes of saccharides. The available theoretical studies of heparin and heparin−protein interactions are limited to molecular mechanics and dynamics calculations.[6,12−15] However, there is still no molecular mechanics force field parametrization capable of adequately reproducing all polysaccharide conformational features.[15]

The present work reports in detail the structural parameters for four DFT-optimized sodium salts of disaccharides (D−E, E−F, F−G, and G−H) of the DEFGH structural unit of heparin and their anionic forms using the B3LYP/6-31+G-(d) method. Of particular interest is the overall shape of sodium salts of disaccharides determined by the conformation of the glycosidic linkage and how this shape changes upon sodium cation dissociation or in a water solution.

2. COMPUTATIONAL DETAILS

The geometries of the sodium salts of the disaccharides D−E, E−F, F−G, and G−H and their anionic forms (Figure 1) have been completely optimized with the Gaussian 03 and Jaguar 6.0 program systems,[16,17] using the density functional theory[18,19] B3LYP/6-31+G(d) method.[20−22] The solvated systems were treated with a self-consistent reaction field method, using the Poisson−Boltzmann solver of Jaguar.[23,24]

The interaction energy, ΔE, for the reaction of a sodium cation with Lewis bases [$Na^+(g) + L^-(g) \rightarrow NaL(g)$] is given by the following equation:

$$\Delta E = E(Na^+ \cdots \text{ligand}) - \{E(Na^+) + E(\text{ligand})\} \quad (1)$$

where $E(Na^+)$ and E(ligand) are the energies of the metal cation and ligand molecules, respectively, and $E(Na^+ \cdots$ ligand) is the energy of the complex. Interaction energies computed using the double-ζ 6-31+G(d) basis set are always, by about 5−10 kJ mol^{-1}, higher than those of the more accurate[25,26] calculations using the triple-ζ basis set. However, the relative trends in the individual stability of metallic complexes are equally reproduced by both methods. Thus, the B3LYP/6-31+G(d) method should be used as a relatively inexpensive alternative for the study of the coordination of cations in oligomeric structural units of glycosaminoglycans.

3. RESULTS AND DISCUSSION

3.1. Molecular Structures. The D−E, E−F, F−G, and G−H disaccharidic units of heparin model the pentasaccharide DEFGH structural units of a typical fragment of heparin, in which two neighboring structural units of heparin bound by the (1−4) glycosidic bonds are substituted with the methyl

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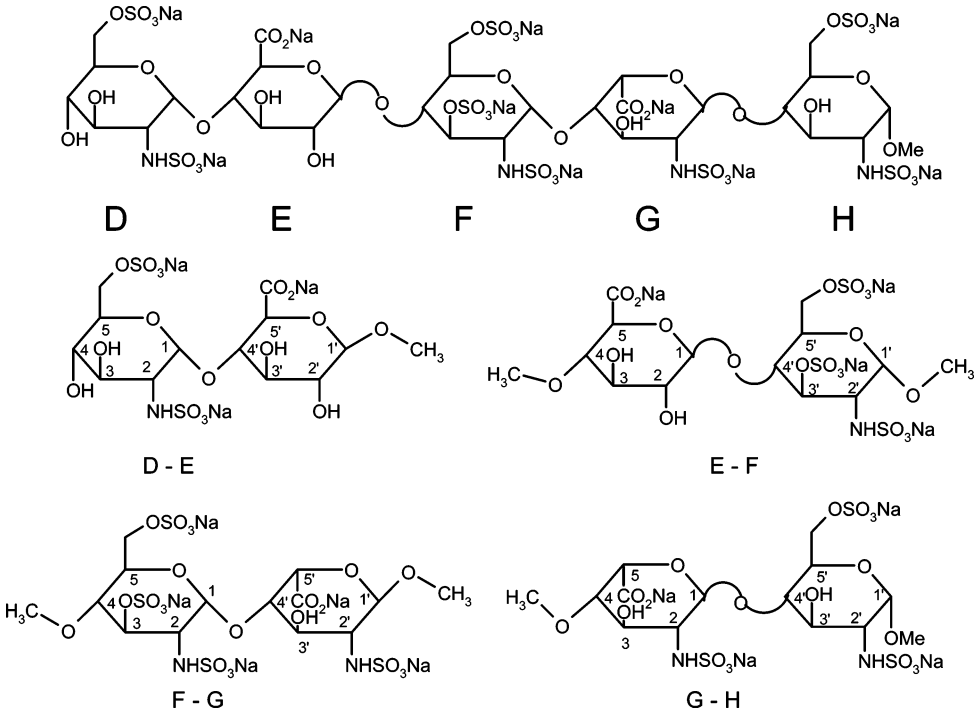

Figure 1. Structure of the biologically active pentasaccharide and the model disaccharides investigated.

groups (Figure 1). The pyranose rings of the D-glucosamine in these dimers were considered in the more stable $^4C_1$ conformation. The starting conformation of the L-iduronic acid building unit G in dimers F−G and G−H was set to the skew-boat ^2S_o form. This conformation was taken as it is the prevalent form of this residue in heparin and its model compounds.[1,6,26] Initial conformations to be used in the DFT calculations of the dimers studied were constructed my means of the Gauss View graphical interface of Gaussian. The relative orientation of pyranose rings defined by two torsion angles (φ, ψ) at the (1 → 4) glycosidic linkage was taken from the experimental data for structurally related heparin-derived oligosaccharides.[27,28] The appropriate number of sodium counterions was added to each dimeric unit at positions derived from previous studies of similar model systems.[29−32] In these systems, the sodium cation symmetrically bridges the two oxygens of the carboxylate and sulfate groups. An examination of the structures of sodium salts of the dimeric units with the Gauss View program indicates that the conformations at the (1 → 4) glycosidic linkage are also preserved in the resulting fully optimized structures.

The Cartesian coordinates (Å) of all gas-phase dimers investigated, optimized at the B3LYP/6-31+G(d) level of theory, are given in Table A of the Supporting Information. To check the correctness of the B3LYP-calculated structures, especially the geometry of sodium bridges, we also performed a full geometry optimization of the dimer D−E, Table A of the Supporting Information, at the ab initio SCF HF/6-31+G(d) level of theory. An examination of the space models of the B3LYP- and Hartree−Fock-computed structures of this dimer shows that both methods give essentially the same results.

The sulfate group in the gas-phase species investigated is essentially tetrahedral with O−S−O angles ranging from 102° to 107°. Only two negatively charged oxygen atoms in the sulfate SO_3^- group (S−O_a and S−O_b) are involved in coordination of the sodium cation. The pattern of these bond lengths in the sodium salts of the dimers studied allows them to be recognized as S−O bond types with considerable resonance bond character. The third (S−O_c) bond in the sodium salt species is, in the majority of the systems studied, not involved in coordination to the sodium cation and may be recognized as a S═O bond type with optimal lengths in the range 1.44−1.45 Å. The S−O_c bond length is similar to that of the typical S═O (1.514 Å) in $(CH_3)_2$S═O computed by us at the B3LYP/6-311++G(d,p) level of theory. Thus, the coordination of the sodium cation to the O_a and O_b oxygen atoms of the sulfate group via the bifurcated bond in neutral sodium salts of the dimers D−E, E−F, F−G, and G−H results in appreciable shortening of the free S−O_c bond. For both carboxylate and sulfate sodium ion complexes, bidendate (direct) bonding was found in the relatively short O···Na separation range (2.2−2.4 Å). The O(a)···Na···O(b) angle of the "bifurcated" sodium bond lies within the relatively narrow interval of 56−65°. However, the overall shape of the molecular structure of the neutral sodium salts of the D−E, E−F, F−G, and G−H dimers is, in the gas-phase, stabilized via the tridendated Na···O sodium bonds (Figures 2−5). The ionization of the respective sodium salts results in structural rearrangements of polyanions.

Dimer D−E. In the dimer D−E, Figure 1, the Na^+ atom of the C(2)−$NHSO_3Na$ group is in coordination with the oxygen atom of the C(3′)OH group. The molecular structure of this dimer is further stabilized by means of the tridendated sodium bond formed between the C(5′)COO^- moiety and the oxygen atom of the C(4)OH group. The third sodium cation symmetrically bridges the two oxygens of the O-sulfo group of this dimer (Figure 2A). Removal of the three sodium cations leads to the structural change of the anionic sulfate and carboxylate groups. The space structure of a trianion of the dimer D−E is, in the isolated state, stabilized via

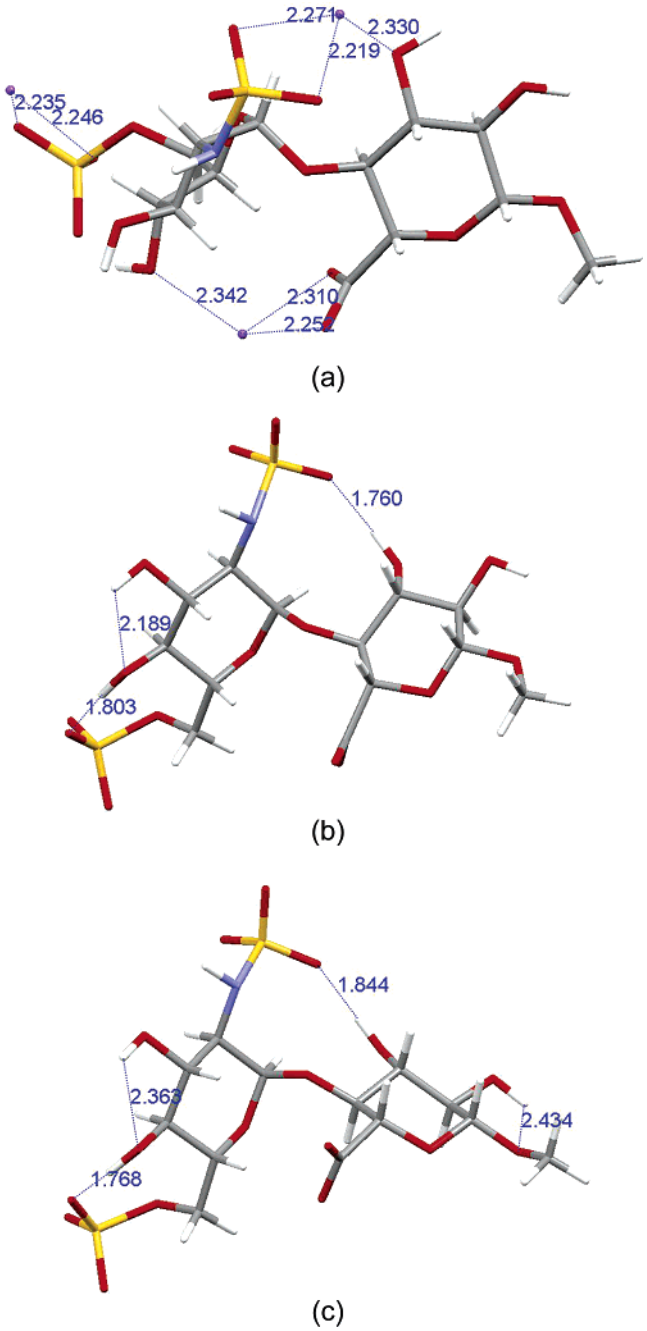

Figure 2. Lowest-energy structures of the D−E disaccharide species. (a) Sodium salt, (b) gas-phase anion, (c) − solvated anion. Lithium cations are purple.

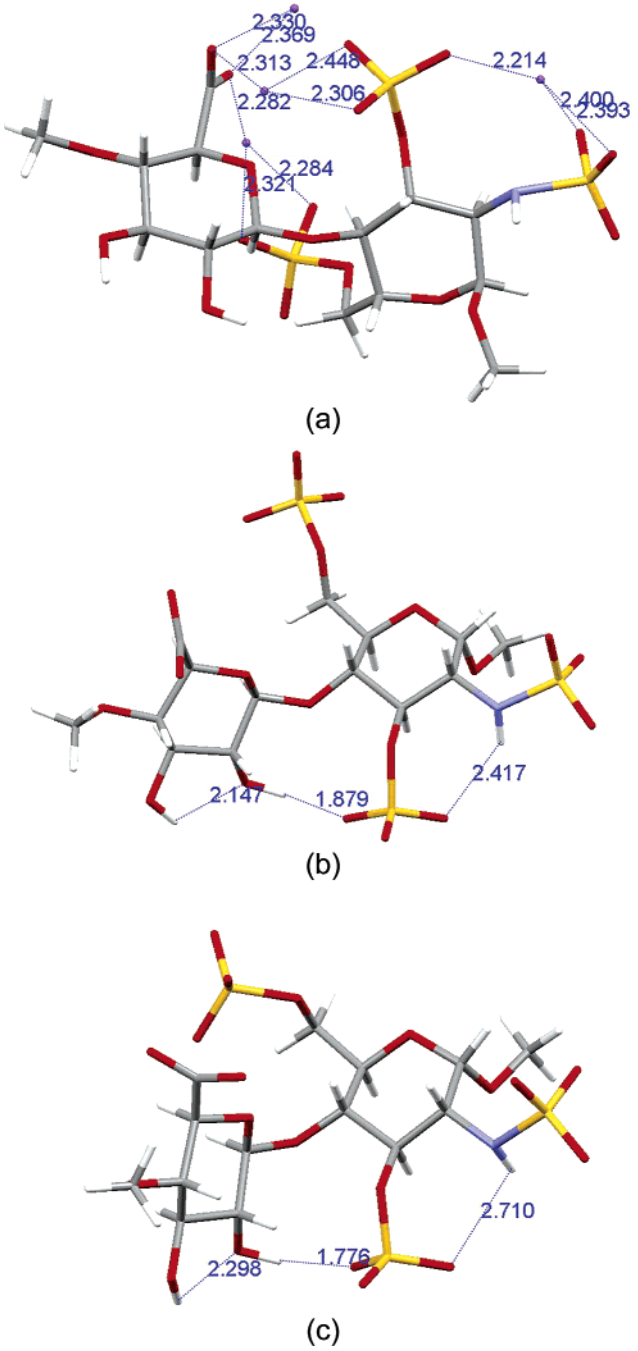

Figure 3. Lowest-energy structures of the E−F disaccharide species. (a) Sodium salt, (b) gas-phase anion, (c) − solvated anion. Lithium cations are purple.

intramolecular hydrogen bonds. All four free OH groups of this dimer are involved in the system of intramolecular hydrogen bonds. The unsubstituted C(3′)OH group takes part in the charged intramolecular C(3)O−H···$^{-}$O−S hydrogen bond. The strong electrostatic attraction in this hydrogen bond results in a much shorter optimal hydrogen-bond distance of 1.760 Å, which is less than the sum of the van der Waals radii[33] of hydrogen and oxygen atoms (2.7 Å). Hydration of this anion caused appreciable geometry changes (Figure 2C). However, the hydrogen-bond pattern observed in the gas-phase dimer D−E was also preserved in the solvated system.

Dimer E−F. Subunit F of this dimer is substituted by three sulfate groups and represents the most sulfated residue in heparin. The minimum-energy molecular structure of the tetrasodium salt of this dimer is, in the gas-phase, stabilized via three multiple sodium bonds connecting the polar groups of E and F pyranose rings (Figure 3A). In addition to this, the structure of the monosaccharide F is stabilized by the system of coordination metal bonds formed between the sodium cation and anionic sulfated groups. The Na^{+} cation of the C(2′)-$NHSO_3^{-}$ group is, via third coordination, bounded to the "free" oxygen atom of the conformationally flexible C(5′)-OSO_3^{-} moiety with an optimal O···Na^{+} distance of 2.214 Å. The gas-phase structure of the pentanion of dimer E−F is stabilized by two intramolecular five-membered hydrogen bonds formed by OH groups of the subunit E (Figure 3B). Hydration of this anion caused a conformational rearrangement of the functional sulfate groups

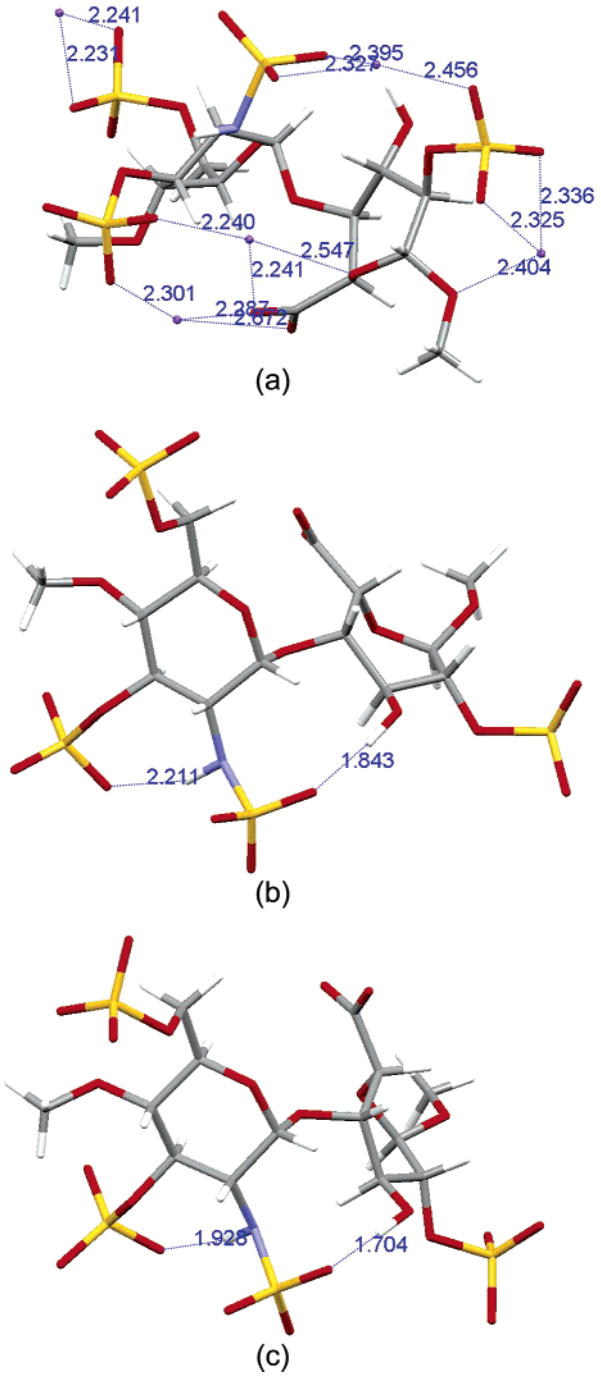

Figure 4. Lowest-energy structures of the F−G disaccharide species. (a) Sodium salt, (b) gas-phase anion, (c) − solvated anion. Lithium cations are purple.

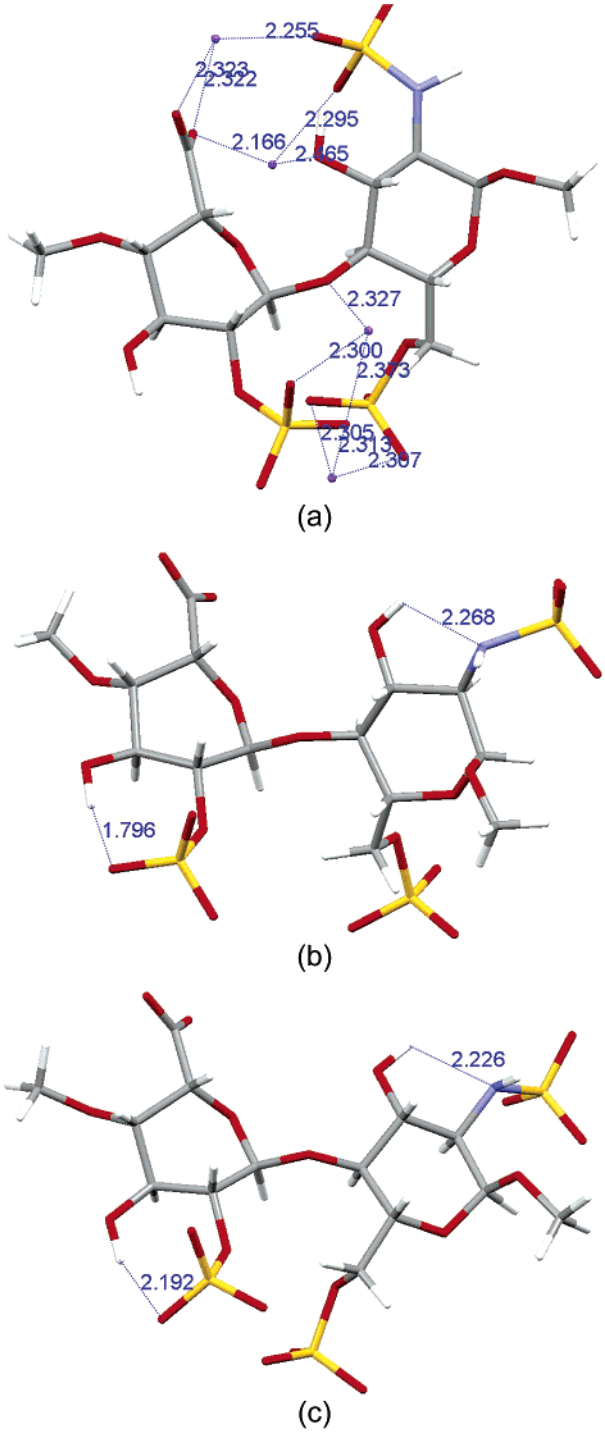

Figure 5. Lowest-energy structures of the G−H disaccharide species. (a) Sodium salt, (b) gas-phase anion, (c) − solvated anion. Lithium cations are purple.

and a change of conformation around the glycosidic bond. The minimum-energy conformation is stabilized by an intramolecular C(2)OH···$^{-}$OSO$_3$C(5′) hydrogen bond with a length of 1.776 Å (Figure 3C).

Dimer F−G. The gas-phase structure of the neutral molecule is determined by the coordination of five Na^+ cations with polar groups of neighboring saccharide rings of this dimer. The sodium cation in the C(6)−OSO$_3$Na moiety is bound by means of the symmetrical bifurcated coordination bond with an optimal Na^+···O length of 2.2 Å. The remaining four sulfate and carboxyl groups are muticoordinated via corresponding Na^+ cations with electronegative oxygen atoms of the neighboring groups (Figure 4A). Optimal Na^+···O distances in these systems are in a relatively large range of 2.2−2.7 Å and reflect a different strength of Na^+ binding to individual polar groups of the dimer. The molecular structure of the pentanion in the gas phase is stabilized via two intramolecular hydrogen bonds. The strong electrostatic attraction in the charged C(2)-NHOSO$_3^-$···H−OC(3′) hydrogen bond stabilizes the conformation around the glycosidic bond. The proton donor NH group of the C(2)NHSO$_3^-$ moiety forms with the C(3)OSO$_3^-$ group a second intramolecular hydrogen bond N−H···O with an optimal hydrogen-bond distance of 1.928 Å. Structural

Table 1. B3LYP/6-31+G(d) Optimized Glycosidic Bond Angles (deg) of the Dimers Studied

dimer	F	Y	Φ, exp	Ψ, exp	Φ_H	Ψ_H	Φ_H, exp	Ψ_H, exp
D−E			89[a]	−141[a]			−49[b](−42[c])	−31[b](−40[c])
			83[c]	−158[c]				
neutral molecule	59.7	−161.0			−56.9	−43.3		
anion	85.8	−157.6			−30.4	−40.3		
solvated anion	59.3	−152.7			−56.4	−34.9		
E−F			−86[a]	−113[a]			43[b](49[c])	12[b](22[c])
			−75[c]	−96[c]				
neutral molecule	−74.5	−98.5			44.3	19.5		
anion	−45.3	−97.2			78.1	29.5		
solvated anion	5.2	−133.2			124.7	−12.7		
F−G			55[a]	−155[a]				
neutral molecule	101.5	172.5			−16.4	−68.6		
anion	80.9	−149.4			−34.8	−29.9		
solvated anion	114.3	−154.4			−1.4	−34.6		
G−H			−70[a]	−115[a]				
neutral molecule	−57.4	−129.7			59.9	−12.2		
anion	−72.1	−95.3			45.5	28.8		
solvated anion	−100.2	−115.4			17.9	5.0		

[a] X-ray data of the pentasaccharide DEFGH bound to the antithrombin.[35] [b] NMR data for free heparin-derived hexasaccharide.[36] [c] X-ray data of the heparin-derived hexasaccharide bound to the fibroblast growth factor.[37]

changes of the F−G anion upon the addition of a solvent (water) are also manifested in the prolongation of both hydrogen bonds (Figure 4C).

Dimer G−H. The geometry of the optimized sodium salt of the G−H dimer is also presented in the Supporting Information. Four Na^+ cations of this dimer are always coordinated with two neighboring polar groups (Figure 5A). Optimal $Na^+\cdots O$ distances are in a relatively short range of 2.2−2.4 Å and result in unique stabilization of the glycosidic bond. A different conformation is found with the gas-phase anion. Every one of the pyranose rings of this dimer carries one free hydroxyl group which is involved in intramolecular hydrogen bonds within individual monomers (Figure 5B). These hydrogen bonds are also preserved in the hydrated anion (Figure 5C).

Structure of the Glycosidic Linkage. For the definition of the torsion angles at the glycosidic bonds, we use the recommendations and symbols of nomenclature proposed by IUPAC.[34] The relative orientation of a pair of dimers studied is described by two torsional angles at the glycosidic linkage, denoted Φ and Ψ. For a (1 → 4) linkage, the definitions of these torsion angles are as follows: Φ = O(5)−C(1)−O(1)−C(4′) or Φ_H = H(1)−C(1)−O(1)−C(4′) and Ψ = C(1)−O(1)−C(4′)−C(5) or Ψ_H = C(1)−O(1)−C(4′)−H(4′). The glycosidic bond angles of the fully optimized dimers D−E, E−F, F−G, and G−H are given in Table 1. This table also contains values of experimentally determined Φ and Ψ torsional angles for the complex of antithrombin with pentasaccharide DEFGH[35] and further available experimental data for glycosidic bond angles in D−E and E−F dimeric units of free hexasaccharide measured by NMR spectroscopy[36] and in the bound state in the complex of hexasaccharide with a basic fibroblast growth factor protein obtained from X-ray diffraction studies.[37]

The equilibrium structure of the sodium salts of the four dimers investigated in the isolated state is determined by the multiple coordination of the sodium cation with oxygen atoms of the sulfate, carboxyl, and hydroxyl groups, respectively. This specific interaction gives rise to the unique overall shape of individual dimers (Figures 2−5) and is manifested by the computed values of the Φ and Ψ torsion angles of the glycosidic linkage. Values of these glycosidic angles for individual dimers are considerably different (Table 1). From the molecular structure of the dimers studied (Figures 2−5), it is clear that the sodium cations are involved in the "screening" of negative charges of disaccharide structural units of heparin. When a positively charged ligand or a protein containing positively charged binding sites (e.g., arginine and lysine residues) binds to heparin, some fraction of the heparin charge will be neutralized, resulting in the displacement of some of the heparin-bound sodium cations in a manner similar to what has been observed for the binding of charged ligands to nucleic acids.[38] In isolated sodium salts of the heparin structural units, sodium cations are involved in "screening" of the negative charges of the polymer, which in turn can lead to the conformational changes in the macromolecule.[32] By the interaction of heparin with proteins, the sodium bonds are substituted with the system of intermolecular hydrogen bonds formed by the sulfate and carboxylate groups of heparin and positively charged arginine and lysine side chains of the proteins.[39] The disaccharides investigated are part of the unique pentasaccharide-binding domain of heparin (also called the antithrombin III binding domain), which is necessary to stimulate antithrombin activity.[11] Thus, the anionic form of the pentasaccharide unique unit of heparin (DEFGH) should be a biologically active conformation observed in the heparin−protein interaction. The analysis of the X-ray structure of the pentasaccharide (DEFGH)−antithrombin complex[35] has shown that the pentasaccharide interacts with antithrombin via hydrogen bonds formed by its sulfates and carboxylates to the arginine and lysine positively charged groups of the protein. The L-iduronic acid residue (subunit G) is, in the bound state, present in the ^2S_o conformation.[35]

The knowledge of the conformational structures of the polyanionic species of these disaccharides may be, thus, important from the point of interpretation of the mechanism of binding of the pentasaccharide domain of heparin to proteins. In the absence of the strong electrostatic forces between sodium cations and disaccharides, values of glycosidic angles of anions in the gas phase differ from those computed for neutral salts by up to 40°. The overall shape

of the dimers D−E and F−G determined by the conformation of the glycosidic linkage is stabilized by means of intramolecular hydrogen bonds. The effect of a solvent (water) on the geometry of the polyanionic dimer species studied was examined using the Poisson−Boltzmann solver of Jaguar. Optimal glycosidic angles of solvated anions differ from those computed for isolated anions by about 10−40° (Table 1). Inter-ring orientations in these dimers are significantly influenced by the coordination of sodium cations, ionization, and solvent effect. In the absence of experimental (gas-phase) data for the dimers studied, the geometry of the parent dimer can be compared only with X-ray data of the more complex pentasaccharide (DEFGH) with antithrombin.[35] The values of the glycosidic angles of this pentasaccharide are also listed in Table 1. In the solid state, the pentasaccharide is bound to the protein in the form of an anion. The solid-state structure of the pentasaccharide can be affected by so-called packing effects, which can distort the structure. In addition to this, the interaction between pentasaccharide and antithrombin can also produce conformational changes. Because both methods are based on different models, the general structural motifs of the pentasaccharide can be compared with results from theoretical methods only. The experimental values for the glycosidic angles in the pentasaccharide−antithrombin complex are well-interpreted by the corresponding angles computed for isolated anions of the D−E and G−H dimeric structural units. In the case of the E−F and F−G dimers, the corresponding experimental values are closer to the structure of the neutral sodium salts. The glycosidic angles for D−E and E−F linkages in the pentasaccharide−antithrombin[35] and hexasaccharide−fibroplast growth factor[37] complexes are in a good agreement, indicating that the heparin polymeric chain binding sites, by the interaction with different proteins, do not undergo large conformational changes (Table 1). The computed glycosidic angles for sodium salts of the D−E and E−F dimers are in good agreement with experimentally (NMR spectroscopy) determined Φ and Ψ torsion angles in structurally related hexasaccharide.[36] The X-ray data for the same glycosidic linkages in the complex of this hexasaccharide with the fibroblast growth factor[37] are slightly different (Table 1). According to our calculations, the inter-ring orientation of both the isolated and hydrated anionic species is quite different. Therefore, it is probable that the biologically active conformation of the unique pentasaccharide unit of heparin should be closer to the structure observed for the parent unbound derivative. Experimental investigations indicate that heparin saccharides show relatively conserved angles Φ and Ψ in glycosidic linkages.[6] Understanding the conformation of individual oligosaccharides of heparin is important when using these residues to study heparin−protein interactions and also in understanding their reactivity.

Accordingly, dimeric saccharide building units of heparin cannot represent all characteristic structural features of the native preparations. To obtain a more complex picture about subtle structural features of the pentasaccharide binding site of heparin, it is necessary to investigate higher oligomers of heparin using sophisticated quantum chemical methods.

3.2. Gas-Phase Sodium Affinities. The calculated gas-phase Na^+ affinities (interaction energies) of the sodium salts of the dimers D−E, E−F, F−G, and G−H are given in Table 2. Table 2 also presents the gas-phase Na^+ affinities for the D−E dimer computed at the higher B3LYP/6-311++G(d,p) level of theory. The interaction energy per Na^+ bond computed using the double-ζ 6-31+G(d) basis set is, by about 5 kJ mol^{-1}, higher than that of the more accurate calculations using the triple-ζ basis set. The B3LYP/6-311++G(d,p) method approximates a high-level calculation with a large basis set.[25,40,41] A comparison of the results from the two DFT methods used shows that the B3LYP/6-31+G(d) density functional theory method performs quite well and can be used as a relatively inexpensive alternative for investigations of larger metallic systems.

Table 2. B3LYP/6-31+G(d) Calculated Gas-Phase Na^+ Affinities in the Dimers Studied (in kJ mol^{-1})

species	total Na^+ affinity	affinity per Na^+	number of Na^+ bonds
D−E	−2167.6 (−2154.9)[a]	−722.5 (−718.3)[a]	3
E−F	−3472.0	−868.0	4
F−G	−4611.1	−922.2	5
G−H	−3359.7	−839.9	4

[a] Becke3LYP/6-311++G(d,p) method.

No corrections for basis set superposition errors (BSSEs) were incorporated into the results, because B3LYP calculations with the larger basis set 6-311++G(d, p) changed the interaction energy only slightly (Table 2). Thus, the basis set used here is adequate to reduce the basis set superposition error effects. Previous calculations[42] of the sodium affinities of organic and biological molecules using the 6-311++G(d, p) basis set indicate that the computed total BSSE is small (less than 8 kJ mol^{-1}).

The computed sodium affinities represent values for the dissociation of several sodium monocations from their binding sites (carboxyl and sulfate groups). For the purpose of comparison of the contribution of the individual Na^+ cations to the interaction energies, the sodium affinity per Na^+ cation is also presented. The largest interaction energy per Na^+ cation was found in dimer F−G. Inter-ring orientation in this dimer is considerably stabilized by the system of coordination bonds formed by three Na^+ ions and functional groups of both rings. Three sodium cations are also involved in the stabilization of the inter-ring conformation of dimers E−F and G−H. On the other hand, in dimer D−E, only two Na^+ cations form inter-ring coordination bonds. The ionization of the sodium salts of the dimers D−E, E−F, F−G, and G−H is associated with considerable conformational rearrangements of ionic species (Figures 2−5). These rearrangements cause additional energetic stabilizations of anionic species. Heparin has been recognized to bind to the receptor proteins mostly through its anionic (carboxylate and N- and O-sulfate) groups of the unique pentasaccharide moiety.[6] The ab initio calculations of the monomeric unit of heparin[26] (1,4-DiOMe IdoA2S sodium salt) have shown that the breaking of the sodium bonds in the glycosaminoglycans is costly in terms of enthalpy but results in an appreciable gain in entropy. It is assumed that much of the Gibbs energy of interaction of heparin and its derivatives with proteins should be derived (like with the DNA) from the entropically favorable release of Na^+ ions.[6] However, the sodium ion affinity of glycosaminoglycans is greatly affected by the site's structural features and stereo effects and the type of intramolecular noncovalent interactions that can be supported at or near the binding group.

More detailed calculations of the interaction enthalpies and Gibbs energies of the sodium salts of the dimers D−E, E−F, F−G, and G−H are also in progress in our laboratory.

4. CONCLUSIONS

This theoretical study set out to determine stable conformations (neutral and ionized forms) of D−E, E−F, F−G, and G−H dimers, for which a relatively small amount of experimental physicochemical data exist, considering their chemical and biological importance. When theoretical methods are used, the following conclusions can be drawn.

The equilibrium structure of the sodium salts of the four dimers investigated in the isolated state is stabilized through the coordination of sodium cations with the oxygen atoms of the sulfate, carboxyl, and hydroxyl groups. This specific interaction gives rise to the unique overall shape of the individual dimers and is manifested by the computed values of the Φ and Ψ torsion angles of the glycosidic linkage. Values of these glycosidic angles for individual dimers are considerably different.

The relative stability of individual anionic species of the D−E, E−F, F−G, and G−H dimers is affected by the solvent (water) and extra stabilization of the pyranose ring conformation by means of intramolecular hydrogen bonds.

The experimental values for the glycosidic angles in the pentasaccharide−antithrombin complex are well-interpreted by the corresponding angles computed for isolated anions of the D−E and G−H dimeric structural units. In the cases of the E−F and F−G dimers, the corresponding experimental values are closer to those of the structure of the neutral sodium salts.

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Supporting Information Available: B3LYP/6-31+G(d) optimized Cartesian coordinates (Å) of all gas-phase dimers investigated. This material is available free of charge via the Internet at http://pubs.acs.org.

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