Nonspecific Electrostatic Binding Characteristics of the Heparin-Antithrombin Interaction

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INTRODUCTION

Heparin (Hp), a sulfated glycosaminoglycan (GAG) isolated from animal organs, has been used clinically as an antithrombic agent since the 1940s.1 For its antithrombic activity, Hp binds to plasma antithrombin (AT) with remarkable affinity, inhibiting a series of coagulation proteins such as thrombin and factor Xa.2 It also binds to numerous other proteins in a manner directly related to the biofunctionality of its structurally simi-
lar relative heparan sulfate.\textsuperscript{3,4} For this reason, and because of Hp’s longstanding clinical use, AT-Hp binding became a paradigm for GAG-protein interactions in general, and the conceptual framework and methodologies developed for this pair set the stage for elucidation of interactions between other GAG-protein cognates, e.g. heparan sulfate-growth factor.

Native Hp chains contain as many as 18 different disaccharides whose sequence arrangement is to a considerable extent randomized by the biosynthetic pathways.\textsuperscript{4} This intrachain irregularity along with the strong intramolecular repulsive forces favor an expanded flexible chain\textsuperscript{5,6} as opposed to a helix. Although there are contradicting opinions about helicity of Hp chains, viscosity-MW relations and effects of ionic strength on hydrodynamic radius\textsuperscript{7} are entirely consistent with the behavior of a flexible polyelectrolyte. Such measurements may not be sensitive to the presence of interrupted helical sequences, and therefore do not preclude a propensity for adopting helical residue conformations in short sequences, as found for aqueous carboxymethylamyllose in the absence of helicogenic agents;\textsuperscript{8} such structures however are readily destabilized by intramolecular electrostatic repulsion.\textsuperscript{8} More significantly, inter- and intra-molecular heterogeneity itself presents great challenges in defining even the one-dimensional structure of Hp, thereby making the elucidation of structure-property relations a formidable task.

While heterogeneity can be reduced by a variety of methods, it is important to recognize that any fractionation—by MW (SEC),\textsuperscript{9–12} charge content (IEC),\textsuperscript{13–15} or ATIII-affinity (AC)\textsuperscript{9,11,12,14–21}—may yield a sample somewhat homogeneous with respect to one characteristic, but broader with respect to others, and the correlations among these characteristics are not entirely clear. The full consequences and significance of the complexity distribution of native/unfractionated Hp remain undisclosed. These unresolved difficulties shifted the focus of structure–activity research to oligomers (i.e. oligoheparinoids) for various reasons: (a) systematic structural variations could be done more effectively with oligosaccharides; (b) access to truly homogeneous samples is only attainable through synthesis of smaller oligomers; (c) oligoheparinoids are clearly more amenable to structural determination, especially with the advent of more powerful and adaptable mass spectrometry and related hyphenated methods; (d) the methods of conformational analysis and NMR methods are applicable to monodisperse oligosaccharides; and (e) complexes of AT with synthetic oligosaccharides can be crystallized.\textsuperscript{22} Finally, the interest in Hp-like molecules as potential anticoagulant drugs lead to, and in turn was stimulated by, the isolation from degraded Hp of “the” pentasaccharide with highest AT affinity.\textsuperscript{23–25}

The reason for the focus on oligoheparinoids was therefore mainly tactical, but this focus fortified the general substitution of oligoheparinoids in place of Hp, both in experiment and conceptually. Despite their merits, oligoheparinoids may not appropriately represent the properties of native Hp. For example, the one-site binding model applied in most oligosaccharide-protein binding studies is liable to be inapplicable to native Hp chains, which are likely to possess multiple heterogeneous binding sites exhibiting different kinetics and affinities.\textsuperscript{9} Similarly, the view of a small restricted site on a protein as suitable for oligosaccharide-binding as for the binding of the native chains is questionable, particularly when long-range electrostatic interactions with fixed charges on Hp are likely to extend beyond the size of the pentasaccharide-binding domain. Finally, glycosylation reduces the binding of AT to Hp, but has a diminished effect on pentasaccharide binding,\textsuperscript{26} raising the distinct possibility that steric obstruction of native Hp chains is reduced for their oligomeric counterparts.\textsuperscript{1}

From the perspective of nonspecific binding of polyelectrolytes to proteins (for reviews see Xia and Dubin\textsuperscript{27}; Cooper et al.\textsuperscript{28}), the dominant long-range electrostatic interactions are influenced by overall polyelectrolyte chain characteristics such as charge density and chain flexibility, and subject to screening by low MW electrolytes. This is in contrast to the binding of small molecules to proteins that can be dictated by short-range forces such as hydrophobic interactions, hydrogen bonding, and salt bridges (the pairing of uncompensated charges within low-dielectric and largely anhydrous protein domains). Consequently, oligomeric ligands may bind to a protein with a geometrically defined bound state that arises from structural complementarities of oligomer and protein; i.e., the energy of the bound state exhibits a steep minimum as a function of ligand conformation and orientation. The substitution of oligoheparinoids for native Hp could lead to underestimation of the entropic penalty of binding and overestimation of enthalpic contributions,\textsuperscript{29,30} and, as noted above could also underestimate steric restrictions on ligand conformation. In considering the two perspectives now introduced, it is worth pointing out that the perception of AT in apo- or holoprotein states is awkward at best when the “ligand” is in fact capable of binding several proteins, so that roles of host and guest are reversed.

While the value of oligoheparinoid studies is incontrovertible, the literature reflects different levels of caution regarding their relevance to structure–property relations of native chains. One example is extrapolation from oligosaccharide studies to conclusions about the rigidity of polysaccharide, with resultant descriptions of native chains as “relatively rigid,” “relatively flexible,” “semi-flexible,” or “helical.”\textsuperscript{4–6,31}
Uncertainties about effect of oligosaccharide length on protein binding affinities are also reported for different proteins, and such studies call into question the perception of an unique high affinity (HA) oligomer sequence for each of the many heparin-binding proteins.

The concept of a well-defined biofunctional oligosaccharide sequence imbedded in an otherwise “inactive” chain is coupled to the strong belief that selectivity of Hp to cognate proteins must arise from specificity, i.e., a high level of complementarity between the Hp “epitope” and a similarly well-defined protein Hp-binding site. Of the numerous points that caution against strict interpretation along these lines, we mention two: (1) Hp functionally binds a remarkable range of proteins, many of which are bound strongly by other GAGs [such as dermatan sulfate (DS) and keratan sulfate] or even by unrelated strongly ionic polyelectrolytes (such as polyacrylic acid), and the hypothesis of hundreds of such protein-specific sites on native Hp is difficult to confirm, classical footprinting techniques being inapplicable. (2) Commercial Hp is in fact the heparinoid fraction from mast cell-rich tissues with the highest anticoagulant activity consistent with reasonable yield (also with the highest level of sulfation), and it is generally believed that only 1/3 of the chains in this optimized fraction contain the nominal AT-sulfation), and it is generally believed that only 1/3 of the chains in this optimized fraction contain the nominal AT-binding pentasaccharide. Its absence in the major portion of chains in this optimized fraction contain the nominal AT-sulfation), and it is generally believed that only 1/3 of the chains in this optimized fraction contain the nominal AT-binding pentasaccharide. Its absence in the major portion of chains in this optimized fraction contain the nominal AT-binding pentasaccharide.5 Its absence in the major portion of unfractionated Hp apparently correlates with diminution, not elimination of activity. Some of these disparate opinions and ambivalent results seen in the vast literature on Hp-protein binding might reflect the desire to deconvolute unresolved questions regarding GAG-protein binding. But the question we raise here is the extent to which protein binding by native Hp is a process involving an entire polyelectrolyte chain rather than a short segment.

To explore this question about the correlation of AT-binding with structural aspects of Hp, we use both normal clinical Hp (14 kDa) and a Hp sample with a MW low enough (5 kDa) to permit constructive, if not exhaustive MS analysis. We demonstrate that this lower MW is high enough to retain the polyelectrolyte properties of unfractionated Hp. Our criterion for such nonspecific protein-PE interaction is the nonmonotonic ionic strength dependence seen for protein binding by a number of polyelectrolytes, both synthetic and biological, some plausibly cognates. Importantly, the ionic strength corresponding to maximum in binding \( I_{max} \) is determined by well-defined but rather global parameters: at \( I_{max} \) the electrostatic screening (Debye) length is equal to the protein radius. This correlation arises from the combination of short-range attraction and long-range repulsion when e.g. a polyanion binds to the positive domain of a globally negative protein (pI < pH), and provides a rather explicit signature for nonspecific electrostatic binding. We employ electrostatic modeling of AT to test the sufficiency of long-range electrostatics in accounting for the results. SEC/MS analysis is applied to AT-affinity fractions of low molecular weight heparin (LMWH) in search of overall structural differences among low, medium, and high affinity fractions. The binding behavior, affinity chromatograms, and charge density profiles by capillary electrophoresis (CZE) of LMWH are also compared to those of another GAG, DS, to examine the level of uniqueness of the AT-Hp interaction. We conclude that generic electrostatic effects play a significant role in dictating the binding of Hp to AT, and infer that complementarity between the charge pattern on a protein surface and the linear array of charges on a GAGs can contribute to selectivity.

**EXPERIMENTAL**

**Materials**

Bovine serum albumin (fatty acid free, Lot 85155438, \( M_w = 68,000 \)) was from Boehringer Mannheim Corp. (Indianapolis, IN). AT was kindly donated by Frank Church (University of North Carolina, Chapel Hill, School of Medicine). Hp (sodium salt, porcine intestinal mucosa, Lot B27591, B35123) was purchased from Calbiochem (La Jolla, CA). LMWH provided by LEO Pharma (Ballerup, Denmark) was prepared by enzymatic depolymerization to yield a product with \( M_w = 5000 \). Chondroitin sulfate B (DS/β-heparin) was purchased from Sigma Aldrich (St. Louis, MO). All other reagents were from Fisher Scientific at the highest available purity. All solutions were prepared with Milli-Q water (Millipore, Milford, MA).

**Methods**

**Capillary Electrophoresis.** Capillary electrophoresis (CZE) was performed using a Beckman P/ACE 5500 instrument equipped with a UV detector or an Agilent HP3D CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array multiple wavelength UV detector. Uncoated fused silica capillaries were employed to prepare columns with dimensions 50 μm × 57 cm (50 cm effective) for the Beckman instrument, and 50 μm × 33 cm (24.5 cm effective) for the Agilent instrument. The new cartridges were rinsed with methanol for 2 h prior to use. The column was rinsed 30 min with 1N NaOH followed by 30 min rinse with Milli-Q water prior to each experiment.

Frontal analysis continuous capillary electrophoresis (FACCE) experiments were performed by equilibrating the capillary with the electrophoresis buffer for 3 min followed by injection of the neutral marker for 3 s, then by continuous injection of sample with a constant voltage. Separation was observed as continuous plateaus corresponding to free protein and protein-Hp complex. After each electrophoretic run, the capillary was rinsed with 0.1M NaOH for 3 min followed by 3 min rinse with H₂O. The experiments were performed with constant concentrations of Hp, LMWH, or DS and varying AT concentrations. The amount of free protein was determined from the height of the free protein plateau using a calibration.
Affinity Chromatography. Fractionation of LMWH according to AT affinity was performed on a gel purchased from Molecular Innovations (Southfield, MI), which was prepared by coupling 65 ml of ATIII at a concentration of 3.5 mg/ml (227 mg) to 43 ml of Affi-Gel 15 resin (BioRad, catalogue No. 153-6052). Coupling was carried out in 0.15 M sodium phosphate buffer (20 mM sodium phosphate, 0.1 M NaCl, pH 7.4) in the presence of an equimolar amount of acetylated Hp to protect the Hp binding site from the coupling agent.

The gel material was packed into an Econo-column (BioRad) with dimensions 1.5 × 10 cm². The column was connected to a flow adaptor, which then was connected to a peristaltic pump to provide constant and stable flow rates of 0.25 mL/min. A gradient mixer (Rabbit) was used for linear salt gradients. The outlet of the column was connected to a Hellma UV-flow cell with 300 μl cell volume for continuous UV-detection at 210 nm. The outlet of the flow cell was connected to a fraction collector when necessary. Before each run, the column was conditioned with 2 bed volumes of running buffer. The samples were injected with a syringe through a 3-way stop-cock attached to the flow adaptor. After injection, the excess material that was not bound to the column was eluted with initial running buffer before the start of salt gradient. The bound material was then eluted with a linear salt gradient from 0 to 0.5 M NaCl.

SEC/ESI-MS. The chromatographic system consists of a Waters (Milford, MA) HPLC system with two Waters Ultrahydrogel 250 (7.8 mm × 30 cm) SEC columns placed in series and kept at 50°C. MeOH-H₂O (4:6) with 30 mM NH₄HCO₃ was used as the eluent at a flow rate of 0.5 mL/min. A photodiode-array detector recording at 232 nm was placed after the SEC columns, followed by a split leading one-third of the eluate to the mass spectrometer. A Dionex (Sunnyvale, CA) 2 mm ASRS-Ultra column run in the autosorption mode and connected to a Dionex CD20 detector, used as a power supply (50 mA), desalted the eluent before it entered the mass spectrometer to avoid the formation of adducts in the mass spectra and improve sensitivity; ESI-MS spectra were acquired in the negative ion mode on a Micromass (Manchester, UK) LCT time-of-flight mass spectrometer. The cone voltage was 10 V and the extraction cone voltage was 1 V. The capillary voltage was set to 3000 V and the source temperature was 110°C. Nitrogen was used as drying gas at a temperature of 350°C and a flow rate of 600 l/h.

Computational Methods. Computer modeling allows the visualization of the electrostatic potential around a protein as a function of pH and ionic strength. This was calculated by nonlinear solution to the Poisson-Boltzman equation via Delphi v.4.0. The nonglycosylated AT crystal structure was taken from the crystal structure of AT-Hp pentasaccharide complex with PDB i.d. 1AZX from RCSB Protein Data Bank (http://www.rcsb.org). The charges of amino acids on the protein were determined using the spherical-smeared-charged model put forward by Tanford and Kirkwood, utilizing the protein titrations curve as explained previously.

RESULTS AND DISCUSSION

The ionic strength dependence of the interaction of AT with Hp and LMWH was determined via FACCE at pH 6 and at 5 ≤ I ≤ 200 mM. This pH was sufficiently above the protein pI of 5.2 to avoid adsorption of AT on the capillary, but low enough to give complexation over a wide range of I. Figure 1 shows the resulting binding isotherms as ν, number of moles of bound AT per charge of GAG, versus [AT]free. The binding strength at each ionic strength can be qualitatively assessed from the initial slopes of these binding isotherms. To obtain more quantitative information, Scatchard plots (ν/[AT]free vs. ν) were fit to the McGhee-von Hippel equation:

\[
\frac{\nu}{L} = K_{\text{obs}}(1 - n\nu) \left(\frac{1 - n\nu}{1 - (n - 1)\nu}\right)^{n-1}
\]

where L is [AT]free, \(K_{\text{obs}}\) (M⁻¹) is the binding constant, and n is the binding site size in number of Hp charge groups. The use of Eq. (1) accommodates the presence of multiple binding sites,
Table I Binding Constants for AT-Hp and AT-LMWH Interaction at pH 6.5 and Various Ionic Strengths

<table>
<thead>
<tr>
<th>I (M)</th>
<th>log K</th>
<th>n</th>
<th>log K</th>
<th>n</th>
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<td>5</td>
<td>4.19 ± 0.02</td>
<td>4.5 ± 0.9</td>
<td>4.22 ± 0.06</td>
<td>1.4 ± 1.9</td>
</tr>
<tr>
<td>10</td>
<td>4.62 ± 0.02</td>
<td>3.7 ± 0.5</td>
<td>4.42 ± 0.03</td>
<td>1.9 ± 0.9</td>
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<td>25</td>
<td>4.77 ± 0.02</td>
<td>5.4 ± 0.7</td>
<td>4.59 ± 0.01</td>
<td>2.7 ± 0.4</td>
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<tr>
<td>50</td>
<td>4.68 ± 0.02</td>
<td>3.0 ± 0.3</td>
<td>4.14 ± 0.02</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>100</td>
<td>4.16 ± 0.06</td>
<td>5.8 ± 2.3</td>
<td>3.81 ± 0.04</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>200</td>
<td>3.81 ± 0.04</td>
<td>5.5 ± 1.7</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

A likely scenario for the native chain,6 The binding constants and binding site sizes are listed in Table I. Figure 2 shows maxima for the binding constants at I = 25 mM for both Hp and LMWH. Similar values for $I_{\text{max}}$ in the range $5 < I < 50$ mM were previously reported for the complexation of four proteins with both synthetic and biological polyelectrolytes, including both cognate15 and noncognate systems.35,40 These maxima arise when proteins bind polyelectrolytes “on the wrong side of pI" and are a consequence of screening at low I of repulsion between polyanion and the globally negative charge, and the screening at high I, of attraction between polyelectrolyte and the local protein positive “patch." The boundary between the repulsive and the attractive regimes is observed near $\kappa^{-1} \approx R_{\text{pro}}$, where $R_{\text{pro}}$ is the protein radius, and $\kappa^{-1}$ is the Debye length $\approx 0.3/\sqrt{I}$ (nm), so that the position of the maximum shifts to larger I for larger proteins.39 $I_{\text{max}}$ for AT-Hp in fact corresponds to $\kappa^{-1} \approx 2$ nm, close to the radius of AT.

The maxima shown in Figure 2 are believed to arise from binding of polyanion to the positive domain of a protein with negative net charge, and this dipole-like charge distribution for AT is shown by the electrostatic potential contours in Figure 3 at the experimentally studied values of I. Somewhat arbitrarily, the potentials are shown at $\pm 0.1$ kT/e; a segment of Hp with a charge of $-10$ located at this surface would experience just enough electrostatic energy to bind.41 The increase in the negative potential domain at $I < 25$ mM is noticeably greater than that for the positive domain; this corresponds to the decrease in the binding constant with decreasing I. Although it is not possible to predict the exact position of bound Hp based on these images, the ability of intensely negative Hp to avoid proximity to negative protein domains is clearly facilitated at $I > 25$ mM. This observation points out an important distinction between native Hp and small Hp oligosaccharides: the latter would not exhibit the repulsive effects. The maximum in K seen also for LMWH indicates that LMWH captures the "polyelectrolyte behavior" shown by native Hp. It should also be noted that the AT structure used for modeling, like the original AT-pentasaccharide crystal structure,22 was not glycosylated. Although one of the glycosylation sites on AT is at the proposed pentasaccharide-binding site, it has been inferred that the presence of the glycan does not invalidate the accepted short-range interaction model.26 The purpose of our modeling is to convey the geometry of long-range electrostatic interactions, even less likely to be influenced by uncharged glycan, a supposition consistent with prior results26 as noted earlier.

The importance of electrostatic interactions in AT-Hp binding was not totally ignored in earlier literature, but one can identify some failures to recognize the dominant effects of long-range nonspecific electrostatic interactions. Desai et al.42 reported the ionic strength dependence of the dissociation constant ($K_D$)—the inverse of the binding constant—for the AT-binding pentasaccharide and its sulfation variants. $K_D$ increased with increasing NaCl concentrations for all pentasaccharides. The $K_D$ values were nearly identical among the disulfated variants, also the case for the monosulfated variants, which all showed much weaker binding to AT than the disulfated ones. These results, which showed insensitivity to the positions of the sulfate group, suggest the dominance of long-range electrostatic interactions. The ionic strength dependence of $K_D$ ($dK/dI$) was nearly identical for all variants corresponding to a given sulfation level, and this result also suggests that various substitutions do not change the nature of the interaction. Since the ionic strength dependence of $K_D$ is correlated with the nature of the interaction (e.g., less pronounced when the interaction comprises a strong H-
bonding component), these pentasaccharide-AT interactions are either all specific or all non-specific, the latter being more reasonable. Nordenman and Bjork\(^4\) fractionated Hp on AT-Sepharose into two distinct pools of low affinity (LA) and HA Hp, and studied the binding of the latter to bovine AT over the ionic strength range 0.15–1.15 M. The binding constant was observed to decrease linearly with increasing ionic strength, leading to the conclusion that electrostatic forces were dominant. The deviations from linearity observed at \(I < 200 \text{ mM}\)—which might have been indications of a subsequent maximum at low \(I\)—did not lead to additional studies due to lack of interest in conditions below the physiological \(I\). Olson and Bjork\(^4\) studied AT-Hp binding using the 40% enhancement in protein fluorescence, which accompanies the interaction. A marked increase in the binding constant was observed with decreased ionic strength, indicating a predominant role for electrostatic interactions.

The binding stoichiometry for both AT-Hp and AT-Tin was calculated as of 0.16 AT per disaccharide from the binding isotherms which supports a model with multiple binding sites on a single Hp chain. Such a model runs counter to the generally accepted existence of a unique AT-binding sequence. In the latter case only those chains that contain this sequence would show binding affinity, so that affinity chromatography (AC) on an immobilized-AT column would show two peaks. Hence, LMWH was fractionated by AC with a linear salt gradient as shown in Figure 4A. The observation of a continuous peak with a continuous linear salt gradient indicates that LMWH cannot be subfractionated into two distinct LA and HA LMWHs. The application of abrupt change in salt gradients,\(^1\)\(^4\),\(^1\)\(^5\) in AC of Hp may force the result of "high and LA fractions." The current result is consistent with earlier studies\(^1\)\(^1\),\(^1\)\(^7\),\(^2\)\(^0\) in suggesting that all Hp molecules exhibit some affinity to AT, and a broad affinity distribution is observed due to the heterogeneity of the chains.

The broad LMWH peak was divided into three fractions that were pooled and somewhat arbitrarily labeled as LA, medium affinity (MA), and HA. Reinjection of the fractions shown in Figure 4B reveal very large overlap, especially for LA and MA. The elution times of the fractions in Figure 4B and their corresponding collection times in Figure 4A are not identical because (1) the applied sample is 500 \(\mu\)l in reinjection, and (2) the collected fractions were subjected to centrifugal filtration that lead to loss of smaller oligosaccharides, hence the contents of the collected and reinjected fractions are not identical. The most significant difference is observed at the highest affinity end of each fraction, i.e. the later eluting ends of each peak. This may indicate that the highest-affinity fractions play a dominant role.

Although there is a significant overlap of the AC chromatograms of the fractions, it is important to verify that these
fractions consist of materials that have differing binding affinity. This can be accomplished by performing quantitative binding analysis by FACCE. The fractions may also be characterized by means of a chromatographic (peak-average) dissociation constant, $K_L$:

$$\frac{1}{V - V_0} = \frac{K_L}{(V_0 - V_m[L])}$$  \hspace{1cm} (2)$$

where $V$ is the peak elution volume, $V_0$ is the elution of a noninteracting ligand, $V_m$ is the exclusion volume, and $[L]$ is the concentration of immobilized ligand. $V_m$ and $V_0$ were determined by running blue dextran and mesityl oxide, respectively. The binding isotherms obtained by FACCE for LA, MA, HA, and unfractionated LMWH (UF-LMWH) at pH 6.5 and $I = 25 \text{ mM}$ are shown in Figure 5. The binding constants, $K_{AC}$ (the inverse of dissociation constant $K_L$ of Eq. (2)) and $K_{FACCE}$, are listed in Table II. It is observed that despite the chromatographic overlap, a significant difference in quantitative binding affinity is observed in the order LA < MA < HA. This observation suggests that $K_{FACCE}$ may be mostly dictated by the late-eluting AC components; and conversely, that the components with low AT affinity in solution still influence the position of the AC peaks from which $K_L$ is obtained. Since binding to the immobilized column is mostly

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & $K_{AC} (\times 10^{-5})$ & $K_{FACCE} (\times 10^{-4})$ \\
\hline
LA & 4.6 & 1.4 \\
MA & 4.9 & 2.1 \\
HA & 5.4 & 3.1 \\
UF-LMWH & 5.3 & 3.8 \\
\hline
\end{tabular}
\caption{Binding constants, $K_{AC}$ and $K_{FACCE}$ for LMWH and its affinity fractions at pH 6.5 and $I = 25 \text{ mM}$.}
\end{table}
through electrostatic interactions, the late-eluting AC com-
ponents may be the most highly charged. Hence we consider
the hypothesis that an abundance of highly charged compo-
nents is the primary origin of high binding affinity, and
therefore turn to CE and LC-MS analysis of these fractions to
strengthen this hypothesis.

The mobility distribution profiles of LA and MA AC frac-
tions are shown in Figure 6, along with that of UF-LMWH.
HA was not included in this analysis because the amount of
material obtained was insufficient for CE. The range of
mobilities alone is remarkable, and attests to the enormous
heterogeneity of the sample (and Hp in general): while the
number of possible sequence arrangements corresponding to
a given mobility could be enormous, no molecules within
one range of mobility could duplicate any of the molecules
within another range. While this breadth is displayed for all
samples, and while the difficulty of recovering large quanti-
ties of sample from AC results in a low signal, it is still possi-
ble to observe a progressive decrease in the relative amount
of low charge density material with increasing $K_{AC}$. One can
in fact see that the region between $\mu = 3.2$ and $4 \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$
is enriched for LA.

A full molecular identification of the components of
LMWH and its fractions is virtually beyond comprehension.
However, a clear picture of the charge content of those com-
ponents is possible using the SEC/ESI-MS analysis reported
by Henriksen et al.\textsuperscript{27} The SEC chromatograms for the three
fractions and UF-LMWH are given in Figure 7. The disap-
pearance of material smaller than decasaccharides is due to
loss during centrifugal filtration. However, the increase in
chain length (LA $<$ MA $<$ HA) for the fractions, all of which
underwent centrifugal filtration, does indicate a correlation
between chain length and AT affinity. To compare the frac-
tions, the mass spectra of the hexadecasaccharide subpopula-
tions (indicated by the slices shown in Figure 7) were chosen
for spectral deconvolution.

![FIGURE 7](image)

**FIGURE 7** SEC/MS chromatograms of HA, MA, LA, and unfractionated LMWH, detection at 232 nm. Bars correspond to hexadecasaccharide region.

![FIGURE 8](image)

**FIGURE 8** Deconvoluted mass spectra for (a) LA, (b) MA, and (c) HA LMWH fractions corresponding to the hexadecasaccharide region.
The deconvoluted spectra for the region corresponding to hexadecasaccharides (shown by rectangular boxes in Figure 7) for each fraction and UF-LMWH are presented in Figure 8. To each of the most abundant peaks, assignments were made in the form \( (X, Y, Z) \), where \( X \) is number of saccharide units, \( Y \) is the number of sulfate groups, and \( Z \) is the number of acetyl groups. It should be noted that this treatment does not give any information about the positions of the sulfate groups and acetyl groups and thus about the presence of different isomers, but does clearly represent the level of sulfation.

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The preliminary results for the species identified with hexadecasaccharide SEC fraction are listed in Table III, and the relative abundances are plotted against the number of sulfate groups in Figure 9. The quantitation resulting from this deconvolution algorithm used has been shown to produce consistent and meaningful results for LMWH \(^{37}\); in any event possible errors introduced into quantitation do not invalidate qualitative comparisons among the patterns of sulfation observed among the affinity fractions. The most significant observation is the low sulfation for LA, which is consistent with the abundance of low-mobility species in the electropherogram. Taken together with the AC results, it suggests that its low affinity is related to the absence of highly charged components that are also strongly retained in AC. The highest sulfated chains varying in sequence distributions hence are likely to determine the average binding affinity to AT, an observation also supported by the finding that the binding constant for UF-LMWH is intermediate between MA and HA. The importance of PE sequence distribution has also been observed in non-specific electrostatic association of other protein-PE systems. \(^{46,47}\)

To further investigate the role of polyelectrolyte properties in the binding of GAGs to proteins, we decided to characterize the AT-binding and charge distribution of another GAG with a disaccharide composition similar to Hp but with lower level of sulfation. DS is structurally similar to Hp and has weak anticoagulant activity, attributed to the inhibition of factors IIa and Xa, but not thrombin. \(^{48}\) The mobility profiles for Hp, LMWH, and DS obtained by CZE are shown in Figure 10. The mobility range of DS lies within the lower mobility range of Hp and LMWH. This implies that DS is missing the molecules at the high mobility end, hence has a lower average charge density than Hp.
The CE and AC results of DS compared with Hp may illustrate one contribution to GAG protein binding. Although DS does not show anticoagulant activity through binding to AT, the AC of DS on AT-affinity column in Figure 11 shows that DS is retained as well as LMWH, while Hp shows the highest retention. The binding constants of DS, Hp and LMWH were obtained by FACCE at pH 6.5 and $I = 25$ mM, conditions at which maximum binding was observed for both Hp and LMWH. Consistent with its lower charge density, DS displayed a lower binding constant than LMWH (Table IV); however, the two AC retentions are similar. Several explanations for the anomalous AC result exits. (1) Although AT coupling was performed in presence of Hp to avoid coupling at Hp-binding sites, immobilization of the protein could reduce its freedom making the Hp-binding of gel-coupled AT different from that in free solution. (2) Since both attractive and repulsive electrostatic interactions between Hp and AT seem to exist, coupling at sites other than the primary binding site can have effects. (3) The weakly cationic nature of Affi-Gel 15, intended to facilitate coupling of acidic proteins, can introduce an “ion-exchange” form of interaction with Hp.

**CONCLUSIONS**

The role of nonspecific electrostatic interactions was investigated for the AT-binding of both intact Hp and low LMMW. The nonmonotonic ionic strength dependence of the binding of AT to intact Hp was seen to be quantitatively identical to that previously observed for a variety of noncognate protein-polyelectrolyte systems, indicating a significant role for long-range, hence nonspecific protein-polyelectrolyte electrostatic attraction/repulsion. LMWH also displayed at the same salt-dependent AT-binding, indicating that this sample retains the polyelectrolyte properties of native Hp. Affinity fractions of LMWH that showed variable AT binding were analyzed by SEC/MS and CZE, both of which showed less highly charged material in the low-affinity fraction. These results are consistent with an AT-affinity dictated by compositional features of the Hp chain that extend beyond the domain of a comparatively short embedded sequence.

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**REFERENCES**


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