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Glycosaminoglycans as polyelectrolytes

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ABSTRACT

One of the barriers to understanding structure–property relations for glycosaminoglycans has been the lack of constructive interplay between the principles and methodologies of the life sciences (molecular biology, biochemistry and cell biology) and the physical sciences, particularly in the field of polyelectrolytes. To address this, we first review the similarities and differences between the physicochemical properties of GAGs and other statistical chain polyelectrolytes of both natural and abioitic origin. Since the biofunctionality and regulation of the structures of GAGs is intimately connected with interactions with their cognate proteins, we particularly compare and contrast aspects of protein binding, i.e. effects of both GAGs and other polyelectrolytes on protein stability, protein aggregation and phase behavior. The protein binding affinities and their dependences on pH and ionic strength for the two groups are discussed not only in terms of observable differences, but also with regard to contrasting descriptions of the bound state and the role of electrostatics. We conclude that early studies of the heparin–Antithromin system, proceeding to a large extent through the methods and models of protein chemistry and drug discovery, established not only many enabling precedents but also constraining paradigms. Current studies on heparan sulfate and chondroitin sulfate seem to reflect a more ecumenical view likely to be more compatible with concepts from physical and polymer chemistry.

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Abbreviations: CS, Chondroitin sulfate; DS, Dermatan sulfate; FGF, Fibroblast growth factor; GAG, Glycosaminoglycan; GM-CSF, Granulocyte-macrophage colony stimulating factor; HA, Hyaluronic acid; Hp, Heparin; KS, Keratan sulfate; PE, Polyelectrolyte; SEC, Size exclusion chromatography; VEGF, Vascular endothelial growth factor.

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1. Introduction

Glycosaminoglycans (GAGs) are flexible linear bio-polysaccharides heterogeneously decorated with sulfate and carboxylate groups. They are ubiquitous on many cell surfaces and in connective tissues, and constitute an important component of the extracellular matrix. Many excellent reviews describe progress in GAG biochemistry [1–4], with notably recent findings on heparan sulfate[1]. As widely noted, the structural characteristics of GAGs involve multiple levels of heterogeneity (Fig. 1): the disaccharide building blocks (iduronic acid or glucoronic acid or galactose and glucosamine or galactosamine), sulfation type (4- or 6-, or exceptionally 3-O sulfation of the sugar rings), sulfation pattern (distribution of sulfates) and the overall chain length [5–7]. The diversity of the GAGs arising from such heterogeneity is a consequence of the non-template driven biosynthesis of these molecules which is nevertheless wonderfully regulated to allow for modifications of GAG structures in response to cell development, disease states and other variables only partly understood. Such diversity also influences physicochemical characteristics that strongly depend on environment such as chain flexibility, viscosity, and compressibility. One consequence of GAG heterogeneity is their ability to interact with numerous proteins. Through such interactions, GAGs, particularly the "heparinoids" heparan sulfate and heparin, regulate biological processes such as cell adhesion, cell growth and differentiation, cell signaling and anticoagulation [3,8]. The structure–function relationships governing these interactions are not well understood. A significant effort has been made to elucidate protein binding by the tools of molecular biology and by detailed structural characterization. In this sustained effort, the recognition of GAGs as polyelectrolytes has not had a high profile.

Polyelectrolytes (PEs) are linear or branched polymers that contain ionizable groups within their repeating units, resulting in charged chains with dissociable counterions in suitable polar solvents like water. Depending on the structural properties of their repeating units, PEs can display various levels of flexibility in solution. PEs do not have a secondary structure, hence they display randomized configurations in solution, denoted as "random coils". While proteins are sometimes described as PEs because some repeating units contain ionizable groups, their unique tertiary structures lead to solution behavior stongly divergent from those of random coil structure. Hence, classification of PEs exclude biomolecules with such defined tertiary structure, but can sometimes include other biomolecules, e.g. DNA and ionic polypeptides, with well-defined helical secondary structures and limited flexibility.

PEs readily interact with oppositely charged surfaces, and a substantial body of experiment and theory describes such polyelectrolyte adsorption and the resultant bound states [9]. The conformational flexibility of PEs allows for interactions with colloidal particles as well as flat surfaces. Particular significance has been attached to the interaction of polyelectrolytes with oppositely charged particles of many kinds including micelles, liposomes, dendrimers and inorganic colloids, with corresponding theoretical analyses [10,11]. PE binding to proteins must follow similar fundamental physics, but is distinctive in that binding occurs readily even when PE and protein have the same net charge. This is a consequence of protein charge anisotropy, allowing PEs to interact electrostatically with regions in which amino acids of opposite charge are clustered. The many aspects and applications of protein–PE interactions have been discussed in several reviews [12–14], while the interactions of GAGs with proteins are also described in detail in the reviews mentioned in the first paragraph, but often within notably different context. The extent to which protein–GAG interactions can be properly considered as a subset of protein–PE interactions is a central theme of this article.

While there is some debate about the propensity of GAGs to form non-transient local conformations, particularly in biofunctional complexes with proteins [15], it is clear that they behave in free solution as statistical semi-rigid (wormlike) chains [16,17]. Along with high linear charge density (the structural linear charge density of heparin exceeds that of any other biopolymer in non-helical state), these features should justify the inclusion of GAGs as polyelectrolytes. Since a rich and influential literature on the polyelectrolyte properties of DNA was already well-established 20 years ago, including the clear recognition of the role of electrostatics in DNA binding to proteins [18], one might ask why physicochemical and biochemical studies of GAGs have not yet followed a similar path towards convergence. Some possible reasons are (1) recognition of the immense importance of GAGs emerged nearly a half-century later than for nucleic acids; (2) the tremendous difficulty of characterizing the structure of GAGs has discouraged physical chemists (with some notable exceptions [17,19–22]) from physicochemical investigations of such ill-defined macromolecules; (3) the substitution of low MW GAGs, particularly low MW heparin–analogs, driven by the desires for both experimental convenience and new drug development, unparalleled in DNA research, has also provided a distraction from the polyelectrolyte viewpoint; and (4) the well-defined helicity of DNA is more consistent with conventional views of macromolecular structure in biology than the conformational irregularities of the native heparinoids.

The need to recognize the polyelectrolyte nature of GAGs was pointed out almost 20 year ago by Jaques et al. [23] who stated that this oversight could lead to erroneous conclusions from experimental data. Nevertheless, limited recognition of GAGs as polyelectrolytes over the following 15 years, presumably related to the obstacles noted above, can be seen from the number of publications that contain keywords "GAGs/Hp" and "protein interactions" and "electrostatic"(Fig. 2), chosen as an indicator of recognition of Hp/GAGs as PEs. Prior to 2007, less than 10% of papers on GAGs met this requirement. The significant increase in this fraction since 2007, indicates a shift in viewpoint. This might be correlated with the leveling off of papers on heparin–protein interactions with a shift to other GAGs, indicating that the non-electrostatic viewpoint was more characteristic of studies with heparin, for reasons that will be discussed below.

To address the roles of electrostatics in GAG biofunctionality, we first compare the physicochemical properties of GAGs with those of other statistical chain polyelectrolytes of both natural and abiotic origins, and then consider the protein binding of such polyelectrolytes vis-a-vis the interactions of GAGs with cognate proteins. This includes examination of the influences of GAG charge sequence heterogeneity and protein charge anisotropy on protein–GAG interactions. These comparisons bring up inconsistencies between the approaches arising from molecular biology and biochemistry vs. those deriving from physical and polymer chemistry. It may be useful to determine the extent to which these differences are semantic or arise from divergent paradigms.

2. The physicochemical behavior of GAGs is identical to those of polyelectrolytes

Should GAGs be considered as manifesting the behavior of polyelectrolytes, irrespective of their biological origin, in similar fashion to

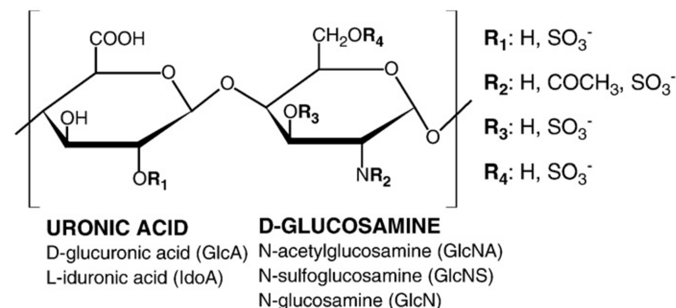

Fig. 1. The main repeating disaccharide unit of heparin indicating possible sulfation patterns at 2,4 and 6 positions.

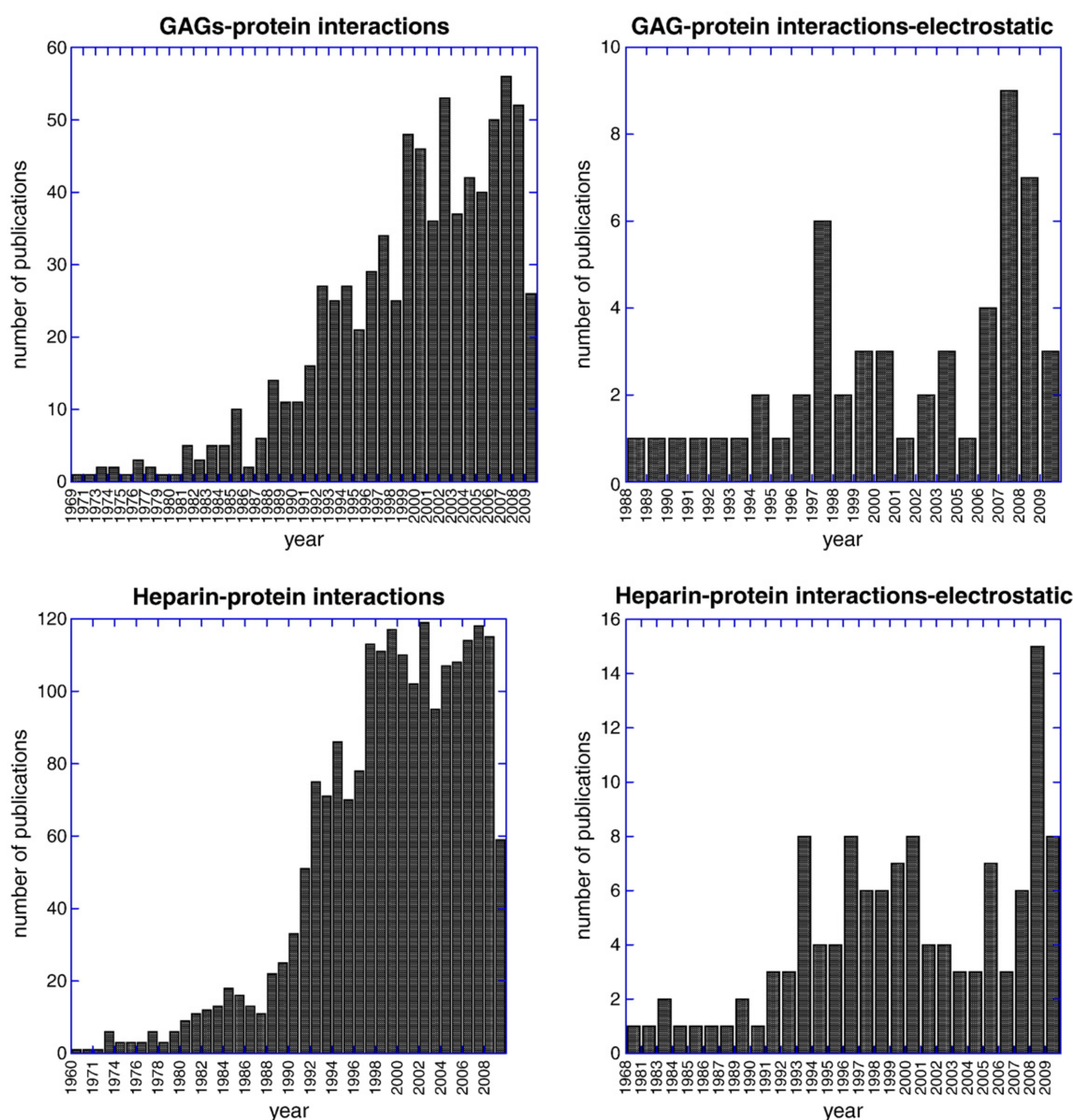

Fig. 2. The number of publications by year which are obtained by searching for keywords: a) glycosaminoglycans + protein interactions, b) glycosaminoglycans + protein interactions + electrostatics, c) heparin + protein interactions, d) heparin + protein interactions + electrostatics (search and analysis performed through ISI Web).

many plant polysaccharides [24]? Do they constitute a particularly unique class of polyelectrolytes, strongly differentiated from the overall group, as does DNA? Or do they differ so markedly from other polyelectrolytes, that such identification is largely irrelevant to structure and function? We approach this question by asking first whether the physicochemical behavior of GAGs is fully explained by the well-established principles and theories governing polyelectrolyte properties.

Established relationships between MW and polyelectrolyte dimensions are central to the characterization of PEs by e.g. viscosity, size exclusion chromatography (SEC), ultracentrifugation and scattering of light, X-ray or neutron radiation. Such studies with GAGs have been inhibited by the concern that samples of different MW would not be strucurally comparable. Pavlov et al. [17] importantly verified that fractions of heparin over a wide range of MW could be treated as a homologous series, essentially identical with respect to composition. The physicochemical properties mentioned above were entirely compatible with the description of heparin as a semi-rigid wormlike chain with bare persistence length on the order of 5 nm. Bertini et al. used SEC with triple detection and found a Mark–Houwink relationship with intrinsic viscosity scaling as MW0.88, entirely consistent with the description by Pavlov et al. Similarly, the radius of gyration of both heparin and dermatan sulfate conformed to the Flory–Fox and Ptitsyn–Eisner equations [16]. With regard to the effect of ionic strength (I), the apparent persistence length of GAGs extracted from bovine nasal cartilage was found to change proportional to $I - 0.50$ by Li et al. [21], who also observed typical effects of I on the second virial coefficient. The influence of I on chromatographic behavior was seen by Guo et al. who found that repulsion between the polyanion and the packing had to be suppressed by $I > 1$ M in order to observe purely steric SEC behavior [25]. Chondroitin sulfate (CS) similarly exhibited the characteristic thermodynamics of polyelectrolyte solutions, with regard to the ionic strength dependence of the counterion condensation [26]. The dissociation of –COOH groups on huyaluronic acid (HA) and CS followed the empirical modified Henderson–Hasselbach equation developed for the pH titration of polyanions, particularly as a function of ionic strength [27]. These authors found indications of random coil chain expansion from potentiometric titration.

The validity of molecular level polyelectrolyte theory for the solution properties of isolated GAG chains has been paralleled by results for brushes and other systems with high segment density. Dean et al. adopted a model of a uniform volume charge density polyelectrolyte

brush to explain the interactions among CS chains [28]. Napa et al. explained the increase in the association of aggrecans (modular proteoglycans composed of 3 protein domains separated by glycosaminoglycans CS and keratin sulfate (KS)), with decreasing pH or salt concentration using a statistical thermodynamics approach previously applied to weak polyelectrolytes, and related this to the ability of these proteoglycans to resist compressive forces in cartilage to electrostatic forces [29].

GAGs have regularly found use in all applications involving "polyelectrolytes" as building materials. Formation of polyelectrolyte complex nanoparticles is shown with the polycation chitosan and heparin (Hp), HA [30] and dermatan sulfate (DS) [31,32] as polyanions. GAGs, especially Hp, are widely used as polyelectrolytes in multilayer build-up [33–35]. Membranes for tissue engineering are reported utilising collagen–GAG complexation, with collagen as the polycation and DS or Hp as polyanions [36]. Hp, HA and CS are also often used as the polyelectrolytic components of hydrogel materials for tissue engineering applications [37,38].

3. Protein binding by polyelectrolytes and GAGs

What are the differences between the binding of GAGs to cognate proteins and protein binding in other systems? The first category includes GAG–protein partners whose interactions, presumably coevolved, are biofunctional, as exemplified by the "heparin-binding proteins" and the heparinoids [8]. The second category refers to the binding of proteins to both synthetic polyelectrolytes and biological but non-cognate polyelectrolytes. It comprises the interactions of "heparin-binding proteins" with polyelectrolytes (including GAGs) not recognized as their biological cognates, and, similarly, the interactions of GAGs with proteins not generally considered biological partners; it also includes combinations of milk proteins with ionic plant polysaccharides, extensively studied in Food Science. Inevitably, such comparisons do not merely involve differences in the systems studied or the techniques chosen, but reflect in equal measure differences between the communities investigating the cognate and non-cognate systems. Differences in the fundamental paradigms of the life sciences and physical sciences strongly influence the questions asked and determine the experiments used to answer them [39].

3.1. Effects of ıonic strength

The effects of pH and ionic strength offer important insights into the nature of the interaction of proteins with synthetic and non-cognate polyelectrolytes [40–51]. While a strong focus on physiological pH and ionic strength may have diminished this approach for "heparin-binding proteins", there has been little doubt that large ionic strength effects reflect a role for electrostatic interactions. [52,53] For the interaction of Hp with Granulocyte-macrophage colony stimulating factor (GM-CSF), the dependence on pH, on the strong influence of GAG sulfation, and the fact that high ionic strength destabilized the interaction all indicated that the association between GM-CSF and GAGs was mediated by electrostatic interactions [54]. Strong salt suppression of antithrombin binding with a high affinity octasaccharide was found by Geurrini et al. with a drop in affinity by a factor of 9 upon increasing the ionic strength from 0.1 to 0.25 M, with the binding of the pentasaccharide Fondaparinux (see below) dropping by an almost identical factor over this ionic strength range [55]. Despite the finding of high and low affinity domains on Hp for selenoprotein, the pH and salt dependence of selenoprotein binding for both high and low affinity Hp suggests electrostatic interactions [56]. Seyrek measured binding constants for heparin and antithrombin [57] and demonstrated the same non-monotonic salt dependence seen for interactions of (1) lysozyme with HA; (2) β-lactoglobulin with a synthetic strong polyanion; and (3) bovine serum albumin with either Hp, or a strong polycation, or synthetic polyanions with or without hydrophobic modification[48]. This special behavior – maximum protein–PE affinity when the Debye length was very close to the protein radius – over such a wide range of system, was presented as a signature of "non-specific" protein–PE systems. It arises from the combination of short-range attractions and long-range repulsions, present of necessity when GAGs bind to proteins at pH > pI (the usual case). It can only be understood in the context of Debye–Huckel ion atmosphere, i.e. when the primary role of counterions is screening of electrostatic interactions between the charged domains of the two macroions. However, as will be discussed below, the ionic strength dependence of GAG–protein affinity may be interpreted in a fashion which actually fortifies the role assigned to specific interactions, and essentially negates the role of screening.

A highly influential paper on the effect of ionic strength, by Record et al. has been applied to GAG–protein interactions. The original paper explained the effect of ionic strength on DNA binding of oligocations [58], based to a large extent on earlier studies by Manning on the condensation of multivalent cations on DNA[59]. In the Record model, binding is driven exclusively by the release of atmosphere-bound univalent ions, salt concentration entering solely by diminishing this entropy. Record used measured DNA-binding constants for a series of oligolysines each containing n ionized lysines and one modified (uncharged and presumably hydrophobic) lysine residue. Assuming that upon "tight" complexation, each residue would release the same number of DNA-bound counterions, one could write:

$$\Delta G^{o}_{obs} = \Delta G^{o}_{\varepsilon} + n\left(\Delta G^{o}_{lys}\right) \tag{1}$$

where ΔG^{o}_{lys} represents an unscreened electrostatic interaction of one cationic amino group with the electric field of DNA, hence independent of screening by [Na+], and $\Delta G^{o}_{\varepsilon}$ corresponds to the interaction of the uncharged modified lysine residue. If the energy of each of these interactions arises from the release of DNA-bound [Na+], then Eq. (1) becomes [60]:

$$\log\ K_{obs} = \log\ K_0 + Z\psi \log\left[Na^{+}\right] \tag{2}$$

where K_{obs} is the measured equilibrium dissociation constant, log K_0 represents contributions unrelated to counterion release, Z is the net charge of the oligolysine, and the number of counterions released per lysine from DNA is $Z\psi$, where ψ is the fraction of counterion Na+ bound per unit charge of DNA. The term log K_0 in the original Record formulation referred to the binding of the modified uncharged terminal residue on the oligolysine, plausibly interacting with DNA through some other mechanism. The factor ψ arises from the assumption that the charged ligand completely neutralizes a stretch of the polyelectrolyte (DNA) that is long enough to itself have the properties of a polyelectrolyte, enabling it to release not only the entire condensed layer of counterions, but also the entire Debye–Huckel screening atmosphere. If it is assumed that the DNA–ligand complex has the same net charge as the DNA with its condensed counterions, then this factor vanishes. While the significance of the model in understanding effects of ionic strength is undisputable, the limitations and consequences of applications to GAG–protein binding will be discussed in the latter sections.

3.2. Effects of pH

The use of pH as a variable provides another way to probe the mechanism of binding. Experiments with non-cognate systems have established the following points: (1) as the charge of the protein moves in a direction opposite to the PE charge, e.g. addition of acid in the presence of a polyanion, the onset of binding can be detected by: a increase in scattering (turbidimetry), a decrease in the mean diffusivity (dynamic light scattering) or a discontinuity in the electrophoretic

mobility (electrophoretic light scattering). (2) This "pHc" [13,42], which depends only on the ionic strength, typically occurs at low salt "on the wrong side of pI" because PEs can often bind to a local domain with charge opposite to both the charge on the PE and the global charge of the protein [61], as mentioned earlier. (3) As protein charge increases in the direction opposite to PE charge, soluble complexes, in which one PE can bind 3–50 proteins, depending on the chain length of PE, exhibit progressive association to form soluble multipolymer complexes. (4) Nearing the point of complex neutrality, phase separation occurs quite abruptly at "pHφ", by precipitation at very low salt, but more commonly by liquid–liquid phase separation (coacervation). (5) The phase states can be represented by boundaries within pH, I, and r space, where r is the protein:PE stoichiometery, and can be expected to broaden with system polydispersity. These effects have been observed for several systems including bovine serum albumin with strong polycations or polyanions [50,62–65], β-lactoglobulin and pectin [41], and analogous behavior has been seen for proteins with adsorbed PEs [47]. Studies outside of physiological pH are less common for GAG–protein systems, and are often neither systematic nor extensively interpreted [54,56,66–68]. It can be noted that efforts to couple experiment with theory while limited do appear more advanced for non-cognate systems [69] perhaps because specific interactions appear almost by definition to be less amenable to reductionist approaches.

3.3. Stabilization or perturbation of protein structure on binding

The stability of protein conformation upon binding to polyelectrolytes has been of considerable interest for both those using synthetic or other non-cognate PEs, and those dealing with cognate systems, but from dramatically different perspectives, in that the first group has generally sought to demonstrate preservation of protein structure and function upon complexation, while the second generally seeks to find evidence of protein conformation changes in the form of allosteric mechanisms for potentiation or inhibition of protein action [70]. Beyond demonstrations involving fluorescence or circular dichroism [71], the preservation of enzyme function has been used by the first group, largely in the context of enzyme immobilization, to establish that PEs minimally perturb protein structure [72–75] and indeed may stabilize it [76–80]. In contrast, the induction of conformational change upon heparin-binding appears almost ubiquitous for a remarkable array of proteins, including serpins [81,82], cytotoxin,[83] apolipoprotein E [84], microtubule-associated protein tau [85] and of course antithrombin [86]. The allosteric interpretation of the influence of GAG binding on protein–protein interactions appears to be predominant, despite earlier suggestions that simultaneous "loose" binding of two proteins could promote their interaction by enhancing encounters [87].

3.4. Considerations of aggregation and phase behavior

The physical states observed for protein–PE complexes corresponding to various degrees of solvation are soluble complexes, coacervates and precipitates [12]. Characteristics of this behavior are sensitivity to pH, ionic strength and protein:PE stoichiometry. A common theme is the tendency towards aggregation and phase separation when complexes approach charge neutrality, and a tendency for the aggregate phases to be truly insoluble when both partners have large charge densities or the ionic strength is low. Given the complexity of the cellular and intercellular milieu, the question of phase state for protein–GAG complexes seems moot or undefinable, but this can lead to in vitro studies in which comparisons may be made between protein–GAG systems that are in solution vs. those in suspension or other colloidal states. Pletcher reported by light scattering very large aggregates formed by association of ca. 100 kDa complexes of ternary ATIII–thrombin–heparin complexes, with different behaviors for bovine vs. human AT, and even precipitation for mixtures of thrombin and heparin[88]. It is not clear why these effects, which might be usefully taken into account in in vitro studies, were essentially never cited.

3.5. Affinities

A central distinction between interactions among proteins and generic PEs vis-a-vis GAGs must be the exquisite regulation of the latter, and it might be expected that higher affinity should be one consequence. Values that have been reported for GAG–protein systems, reported in Table 1 for native Hp with antithrombin, range from 0.15 nM [89] to 20 nM [90]; 2–6 nM for Hp with fibroblast growth factors, FGF-1 or FGF-2; and 40–80 nM with vascular endothelial growth factor (VEGF) [91]. While the range of Kd for non-cognate PE-protein pairs is obviously much greater, there are a number of examples of similar affinities: RNAse + Hp (8 nM) [92]; Lysozyme + HA (10–20 nM) [93]; and collagen + CS (5–40 nM) [94]. If regulated structure of GAGs was to lead to particularly high affinites, we would expect to find that perturbations in the cognate protein would diminish binding. If such regulation would refer to the pentasaccharide unit (see below), we would expect to see the retention of high affinity with pentasaccharide–mimetic oligosaccharides. However, Arocas [89] found a 10-fold reduction in Kd for a low molecular weight Hp drug, Arixtra®, compared to native Hp (and subsequently a 2–4-fold reduction[90]), and Schedin–Weiss found that mutations of antithrombin could significantly enhance binding [95,96].

3.6. Description of binding and the bound state

Comparing biochemical vs. physicochemical descriptions of polyelectrolyte–protein affinity, one cannot find a greater discrepancy than in the understanding of the bound state.

3.6.1. Ligand and substrate

The biofunctionality of enzymes and other proteins involves their binding of small molecules which are recognized and bound with high affinity by the protein host, with well-defined stoichiometry. Since polyelectrolytes frequently have effective hydrodynamic radii in the range of 10–100 nm, they can and do bind many proteins, reversing the host–guest relationship. While the same formalism of binding isotherms (Scatchard, Hill and McGhee-von Hipple plots) apply equally, stoichiometry is likely to be more variable. When the ligands are large proteins, binding cooperativity involves their spatial requirements. Attempts to model GAGs as essentially chain fragments represent an effort to restore the more familiar paradigm of protein–ligand (as opposed to protein as ligand) binding.

3.6.2. Rigidity vs. flexibility

There is a growing recognition that the conformations of proteins in solution and in vivo may show temporal variations from crystal structures, but the perception of a narrow range of energy-minimized configurations for protein–ligand complexes is generally applied to complexes of proteins and GAGs. This view is antithetical to descriptions of polymers in solution, for which entropy dictates a virtually infinite set of configurations with nearly equal energies, all

Table 1
Dissociation constants, K_d, for some protein–GAG pairs.

Protein–PE pair	K_d (nM)	Ref.
Hp–antithrombin	0.15–20	[89,90]
Hp–FGF1/FGF2	2–6	[91]
Hp–VEGF	40–80	[91]
Hp–RNAse	8	[92]
HA–lysozyme	10–20	[93]
CS–collagen	5–40	[94]

measured properties representing averages over space or time. Configurational entropy favors the "random coil" conformation vis-a-vis extended or collapsed chains. While some polymers do crystallize, helices or other similarly ordered structures are unknown for synthetic polyelectrolytes in solution, or for polymers with irregular sequence arrangments. Polyelectrolyte–protein complexes are thus considered to partake of the same temporal variations.

As crystallography is a foundation of protein chemistry, the precise atomic arrangment elucidated from crystal structure is central to the understanding of the protein–ligand complex. Thus, protein–GAG complexes are often protrayed as permanently rigid. This requires perceiving the GAG itself as rigid, usually accomplished by suggesting local helicity [97]. A particularly influential report [98], based on measurements made with a synthetic, hence pure oligosaccharide has helped to promote the notion of helicity in heparin, although this is not easily reconciled with the irregularity of GAG sequence arrangments as well as their high charge density. An additional component of the atomistically fixed view is the tendency to replace full-length chains by oligomers.

3.6.3. Oligosaccharides in place of native chains

The characteristic length scales probed by measurements on polymer solutions are on the order of tens of Å to tens of nm, and graphic representations of poyelectrolytes complexed with other polyelectrolytes or with colloidal partices reflect this. In contrast, protein biochemical processes often reflect events at length scales an order of magnitude smaller, as does the domain of crystallography. In order to avoid the multisite character of native chains and reduce the complexity of their immense heterogeneity, the native chains have often been replaced by oligosaccharides isolated by degradation of native GAGs. The range of oligosaccharide libraries has been enlarged by combinations of chemical desulfation and enzymatic modification with recombinant sulfotransferases [99,100] and further expanded by chemical synthesis [101]. Such libraries can be subject to screening via immobilized proteins [102] or themselves form microarrays[103,104].

While this focus on oligosaccharides has suggested routes to new drugs, and has greatly simplified problems of purification, crystallography and characterization (and perhaps oversimplified docking and MD simulations [105–107]), serious caveats associated with this approach to protein binding, as clearly stated by Powell et al. in 2004, include: overemphasis of enthalpic contributions, and disregard of the loss of chain configurational entropy [89,90,95,96]. Attempts at docking for protein–GAG interactions are thus complicated by the fact that the dimensions of native chains of heparin or heparan sulfate may be similar to or greater than those of the proteins to which they bind. It has also been perceptively noted that docking studies for GAG–protein interactions are severely challenged by "the weak surface complementarity, the high charge density of heparin, and the highly flexible nature of the GAG chain" [108], and these concerns are only partially addressed by substituting oligoheparinoids for native chains. A serious problem that could arise from the use of short oligomers is the absence of long-range electrostatic interactions between the protein and distal units of the bound GAG.

3.6.4. Considerations of long-range interactions

The fundamental properties of polyelectrolytes arise from unrelieved electrostatic repulsion between nearby segments and transient interactions among groups distant from each other along the chain, the latter subject to screening by small ions. Polyelectrolyte structure and properties cannot be separated from these concepts. In protein chemistry, many phenomena such as folding, catalysis, ligand binding and recognition are sucessfully explained by short-range directional (H-bond) and non-directional (hydrophobic) interactions, and coulomb forces between uncompensated charges in high-dielectric domains (salt bridges). A vivid example of the emphasis on short-range interactions is provided by the deptiction of the antithrombin–pentasaccharide interaction via H-bonds by van Boeckel et al. [109], and by a more recent and influential [110] representation of antithrombin binding to the chemically pure heparin analog drug, Fondaparinux (Fig. 3). Short-range specific binding via directionally specific interactions – both of those assigned as hydrogen bonds or unspecified – and both "probable" or "possible" – are represented in Fig. 3b as taking place between the oligosaccharide O-sulfates (without consideration of the limitations on H-bond lengths, bond angle constraints, the intervention of hydration, and geometric constraints). Notably, the van der Waals image (Fig. 3a) portrays the pentasaccharide as residing in a continuous positive potential domain, while the schematic represents a set of pair-wise interactions, the former more consistent with the likelihood of both intramolecular and translational motion of the bound pentasaccharide. The admission of long-range electrostatics into the schematic of Fig. 3b would be equivalent to multiplying the number of lines ad adsurdum.

The fact that electrostatic forces are long-range may be unhappily confused with the notion that they are not also short-range; and since only short-range forces are considered relevant to much of protein chemistry, long-range electrostatic forces are not considered major players in protein structure and function. In the case of their interaction with GAGs, attention confined to the interaction of a locally positive protein binding site with a short segment of GAG is equivalent to disregard of repulsive interactions between the heavily sulfated vicinal GAG units and the globally negative proteins, virtually all significant GAG-binding proteins having $pI<7$. Put differently, since charge sequences on GAGs are clearly non-uniform and protein charges are anisotropic, the complementarity of those two charge patterns should not be ignored. Long-range interactions must also be considered when GAGs play the role of modulating protein encounters.

4. The heparin–antithrombin paradigm

Although the last paragraphs used synthetic heparin oligomers to illustrate tendency to parameterize GAG–protein interactions in terms of short-range forces, it is worthwhile to describe the history and context of a particular group of heparin-like oligomers to understand their powerful influence on such paradigms. Indeed, the estrangement between the (bio)physical chemistry of protein–PE interactions and the glycobiology of GAGs is largely attributable to the course of heparin clinical and pharmaceutical research, which played a leading role in framing the current debate about specificity. The anticoagulant (antithrombic) acitivity of heparin, leading to its clinical use as an FDA-approved drug in the 1940s [111], arises from its ability to bind with remarkable affinity to plasma antithrombin (AT) which functions as a potent anticoagulant, inhibiting a series of coagulation proteins such as thrombin and factor Xa. The isolation and purification of AT in 1973 were followed by extensive studies at multiple research sites between 1976 and 1983, leading to the concept of a unique sequence responsible for AT-binding along with a corresponding molecular level description of the resultant complex. The concept of such a "natural pentasaccharide" as termed by Petitou in 1993 [109] grew out of a drug development program at Sanofi in the 1980s, with the objective of new low MW heparin–analogs, coming to fruition with SANORG™ [112], and Fondaparinux (Arixtra™) [109]. The strategy was a Herculean synthetic cum bioactivity screening program based on an interpretation and extension of earlier observations in 1976 [113]: that gradient salt elution (e.g. from 0.2 to 3 M NaCl) on an antithrombin column produced heparin fractions of different column affinity. Interpreting "were separated into three peaks" as "eluted as three separate peaks", it was inferred that the early elution of some components of heparin demonstrated their lack of AT-binding competence, caused by the absence of an essential recognition sequence. Cautionary notes have pointed out that "The division between the two fractions thus is arbitrary and only dependent on the conditions selected for the affinity–chromatography experiment" [114],

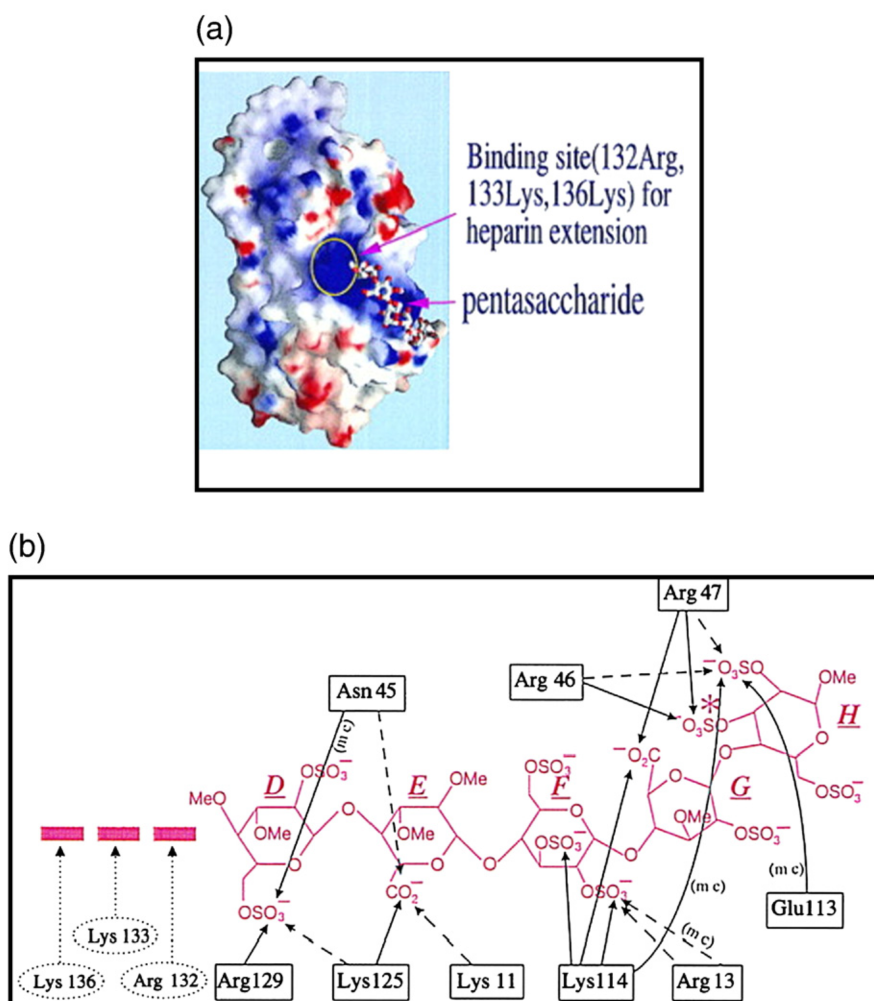

Fig. 3. Representations of a pentasaccharide-antithrombin dimer complex. (a) Superposition of pentasaccharide outline on electrostatic potential surface (blue is positive); (b) hydrogen bonding between pentasaccharide and residues of antithrombin (from Ref. [110]).

and also that "differential ionic strength elution of oligosaccharides may not reflect selection based on their true relative affinities" [2] — referring in other words to the possibility of a contribution of ion-exchange, i.e. electrostatic chromatography. Nevertheless, the interpretations in 1993 of the 1976 results are the basis of the off-cited conclusion that 2/3 of heparin molecules are unable to promote the inhibition of the protein factor Xa (FXa) by AT, and thus lack an essential recognition sequence. In search of this moiety, the Sanofi group first established a minimum heparin length of octasaccharide (dp8) required for FXa activation. To further lower the MW requirement, the hexasaccharide unit "CDEFGH" was identified as a sequence common to the most active of these octasaccharides [115]. On the basis of minimal changes in antithrombic activity observed when the terminal (non-sulfated) residue C of the hexasaccharide was altered, the search was further narrowed to a pentamer "active sequence": GlcNAc/NS(6 S)–GlcA–GlcNS(3 S,6 S)–IdoA(2 S)–GlcNS (6 S), best denoted as D*EFGH, it being in fact a synthetic "variant" of DEFGH, a "natural pentasaccharide" segment. Despite these ambiguities, compounded by the continued finding of "extensions" with higher ATIII affinity than D*EFGH (e.g. Idraparinux™ with 100X greater AT-affinity than Fondaparinux™ [116]) references to the "natural AT-binding sequence of heparin" continue to abound [116].

Despite the fact that this uniquely highly active pentasaccharide D*EFGH was hailed in 1993 as "the unique AT-binding domain of Hp" and the related conclusion that "Hp binds to AT only through a specific pentasaccharide"–subsequently accepted widely without question (see http://en.wikipedia.org/wiki/Heparin) – one may question its position as a logical consequence of the Sanofi findings. Nevertheless, the pentasaccharide model gained considerable credibility with the publication of a crystal structure of the complex between ATIII and a different synthetic pentasaccharide containing an additional N-sulfate on residue "D" [99]. Although this O-alkylated, over-sulfated pentasaccharide was designated by the Sanofi team as a "non-GAG analog", the crystal structure and its interpretation (Fig. 3b) were taken as validation of the specific interactions responsible for the unique AT-binding features of what became known as "the pentasaccharide" D*EFGH. Although further "extensions" of D*EFGH have shown enhanced bioactivity, influence of its "discovery" is still strong in the form of the a priori hypothesis: the precise arrangement of saccharide substituents is central, so that the activity in the presence or absence of one given substituent (e. g. the 3-sulfate group in unit F) could be used to rule out (or conversely, establish) the essential and unique role of that substituent in biological recognition or activation.

The influence of the pentasaccharide paradigm extends beyond the Hp–AT pair. The concept of a "minimum size of Hp and HS sequences required for binding and activity" remains extant as cited in Powell et al. [2]. There is certainly a range of opinions, encompassing the belief that such interactions are not highly specific, although they may be of

high affinity [39]. These differences can be exacerbated by semantic ambiguities such as misleading definitions and classifications of GAG–protein interactions. For example, a review paper [117] classifies GAG–protein interactions in 3 groups: 1. non-specific (any sulfated polysaccharide interacting with any basic protein), 2. strongly specific (Hp with AT) and 3. of intermediate specificity (HS with growth factors). Within each class, "specific" can refer to the group, i.e. "specific protein–GAG systems", to structure, i.e. "specific pattern of sulfation", or more generally "specific oligosaccharide composition". This broad usage creates challenges to clarity.

5. Challenges to the pentasaccharide paradigm

Apart from the difference between high affinity oligoheparins (e.g. Arixtra®,) and native Hp noted above, a number of observations have called into question the model of an embedded oligosaccharide moiety that interacts with a cognate protein via a set of atomistically well-defined short-range forces (i.e. specificity) leading to high affinity (selective) binding. As noted above, Kd values for non-cognate, hence non-specific, protein–PE pairs are not universally lower than those found for ATIII–Hp, so that high affinity alone does not demonstrate specificity. In any event, "it is difficult to infer mechanism from affinity" [2], and changes in affinity can be confused with changes in specificity [39].

A number of challenges confront the proposal that lock-and-key fits between Hp and its cognate proteins underlie biofunctionality. A very large number of polyanions of a remarkable variety appear as Hp-mimetic, either by binding proteins that are Hp cognates, or more often, by producing a biological response resembling that of Hp [39]. Some examples of the former are reported by Joshi et al. [118], who found by isothermal titration calorimetry (ITC) very similar somatotropin binding energies for DS vs. Hp; by Charef et al. who obtained a thrombin binding constant $K_d = 14$ nM for carboxymethyl sulfated dextran [119], and by Desai et al. who found AT-binding with sulfated lignins [120]. Examples of Hp-mimetics go back as far as Heuck et al. who found similar antithrombic effects of potassium poly (vinylsulfonate) and Hp in ternary polyanion/AT/thrombin mixtures [121]. More recent examples are: Hp-like inhibition of cathepsin G by carboymethylated or sulfated dextran [122]; the ability of sulfated fucans to block the complement system of the immune response with activity equal to or greater than GAGs [123]; and anticoagulant activity of sulfated silk fibroin [124] and sulfated lacquer polysaccharides [125,126]. The Hp-like inhibition of angiogenesis also has been observed with alginic acid oligomers, fucoidin, and dextran sulfate [127]. It appears problematic to interpret biological responses observed with heparin as implicating the specific interaction of heparin with key proteins, when non-coevolved and even quasi-synthetic polyanions produce the same response. A related observation is that high affinity binding with heparin is not confined to heparin-binding proteins, as pointed out in Table 1 for RNAse-Hp binding.[92].

The notion of a tightly bound "active site" embedded in an otherwise loosely- or non-bound GAG chain becomes less tenable for progressively larger putative binding sites. For example, Lookene et al. stated that "decasaccharides are the shortest Hp fragments that can completely satisfy the Hp binding regions in dimeric lipoprotein lipase (LPL)" [128]. On the basis of a computational model, Stuckey et al. [129] concluded that a hexadecasaccharide or an octadecasaccharide Hp fragment would provide the minimal length to fully wrap around (the ring of positive charges characteristic of) platelet factor 4 dimers. Such an observation demands considerable variability in this 7 kDa unit (so that a reasonable fraction of native chains might bind), and consequently, the notion of specificity can be preserved only by an ingenuous division of the bound GAG into specifically and non-specifically bound saccharides.

Affirmations that a GAG interacts with a particular protein "through" a designated embedded oligosaccharide are also called into question when "extensions" of that oligosaccharide lead to increased affinity. Guerrini et al. found that elongation of the AT-binding pentasaccharide Fondaparinux™ to form several octasaccharides increased AT-binding by as much as an order of magnitude [49]. These authors mentioned the possibility that nonspecific interactions might indeed be implicated by these results, but on the basis of ionic strength dependence of affinity, NMR studies and docking simulations, they concluded that the pentasaccharide–AT contacts (i.e. specificity) are maintained with additional indeterminate nonionic interactions. Other extensions are not favorable, as reported preparation of a decasaccharide containing the "AT-binding pentasaccharide" displayed a Kd of 290 nM (and also a relatively low ΔH of binding) [130]. Most of the extensions discussed however resulted in enhanced affinity relative to the pentasaccharide; interestingly some that place a non-sulfated saccharide next to the reducing end could give an order of magnitude enhancement[55].

6. Modified and competing views of specificity

We noted above bibliographic evidence of increasing recognition of the role of electrostatics in GAG–protein interactions. This might signal a more nuanced view of specificity, or contrariwise, an apartheid in which specific (providing recognition) interactions are separated from – and in a sense elevated above – non-specific interactions which provide a sort of directionless motive force for binding. A number of investigations suggest the former. Deakin et al. studied the binding of HS/Hp tetrasaccharides containing only two sulfates to hepatocyte growth factor/scatter factor (SF), and found similar affinity regardless of whether those groups were N-, 2–O-, or 6-O–sulfates [131], also finding from NMR chemical shifts that these oligosaccharides are bound to the same site. They inferred the absence of "any apparent positional requirement for sulfation", concluding that "an unexpected degree of flexibility in the GAG–HGF/SF interface" allowed a single protein binding site to "accommodate iduronate-containing sequences of variable sulfation patterns and/or density from different GAGs". This observation was in accord with an earlier finding from photoaffinity labeling studies, that "small polyanionic ligands can be bound in multiple orientations in the polyanion-binding site [of FGF-1 and FGF-2]" [132]. Catlow et al. also used the FGF/SF interface to compare both affinity and activity from (1) 2,6-O-sulfated DS species from Ascidia nigra, (2) mammalian (mainly 4-O-sulfated) DS species, and (3) specifically desulfated heparins. They found that "no specific sulfate isomer, in either GAG, is vital for interaction and specificity". Moreover, different GAGs of similar sulfate density had comparable properties, with affinity and potency increasing with increasing sulfate density [133].

The studies just mentioned strongly indicate a correlation between "non-specific" electrostatic binding and activity. Such a correlation suggests that non-specific electrostatic interactions can play a role in recognition. Results from outside of glycobiology provide some support for this provocative suggestion: Beals et al. examined the effects of modification of ten cationic residues in sites 1 and 2 of erythropoietin (Epo) on kon and koff with erythropoietin receptor (EpoR) [134]. Point mutations to alanine or glutamate produced a 2–5 fold drop in kon and concomitant drop in activity, proportional to the change in charge, but regardless of mutation to alanine or glutamate, and regardless of the mutation site (1 or 2). This behavior was construed as a global electrostatic effect on what would be considered a "specific" interaction, implying that nonspecific interactions might be viewed as modulating selectivity. This work was complementary to a later study by the same group of the effect of ionic strength on the suppression of kon by glycosylation, which was then inferred to be evidence of long-range electrostatics. It was found [135] that the dependence of kon on ionic strength for five isoforms of Epo conformed well ($R2 \geq 0.96$, $0.05 < I < 1.0$ M) to a linear dependence of ln kon on $(1+\kappa a)$ 1 where a is the distance of closest approach of the two proteins, and κ is the Debye–Hückel parameter, proportional to

I1/2. This dependence is in accord with the treatment of Schreiber et al. [136] which leads to:

$$\ln k^0_{\rm on} = \ln k^0_{\rm on} - \frac{U}{RT}\left(\frac{1}{1+\kappa a}\right) \quad (3)$$

where U is the hypothetical salt-free electrostatic interaction energy, and kono is kon in the limit of high salt when all interactions are screened by counterions, a relationship demonstrated for a number of cognate pairs, including hirudin/Thrombin, interferon α2/receptor, and barnase/barstar [137,138].

A dramatically different interpretation of the salt effect was introduced above in Section 3.1 as evolving from a series of papers by Record and co-workers [60,139,140] for the binding of oligolysines to DNA, and is often expressed as a modification of Eq. (2):

$$\log K_{\rm obs} = \log K(1M) + z\psi \log \left[Na^+\right] \quad (4)$$

where $K_{\rm obs}$ is the equilibrium dissociation constant, z is the charge number of the oligocation and ψ describes the degree of counterion binding to DNA. The 2nd r.h.s. term of course vanishes in 1 M salt, in a manner unrelated to screening. The remaining term, obtained by extrapolation of log Kobs to [Na+] = 1 M, and previously written in Eq. (2) as log K_0, continues to come from the release of counterions from the condensed layer. In the original Record treatment, log K_0 arose from $\Delta G^{\circ}_{\varepsilon}$ in Eq. (1) and described the interaction of the modified oligolysine nonionic group with DNA, but later came to signify all "nonionic interactions" regardless of the presence of any such group. It was thus assumed that extrapolation to 1 M added salt would provide "the nonionic component of the interaction", i.e. the total interaction minus "The polyelectrolyte effect". Since the Record model, based on "tight interactions" between polyelectrolyte (DNA) and guest oligoion, gave a linear dependence of log $K_{\rm obs}$ on log[Na+], it was subsequently assumed that observation of such linearity was prima facie evidence of tight binding (absence of ion atmosphere effects): "Since the effects of salt on Hp-protein equilibria are due mainly to differential binding of cations to the Hp, and anions to the protein, it is inappropriate to interpret these effects in terms of simple ionic strength or screening effects" [53]. The log $K_{\rm obs}$/log[Na+] criterion came to be applied somewhat liberally despite the notorious ease with which log-log plots display linearity particularly when the independent variable ranges over less than an order of magnitude. The striking difference between Eqs. (3) and (4) reflects a fundamental difference in the model of binding.

While Record encouraged the extension of Eq. (2) from DNA–oligocations to DNA–protein systems, Lohman and Mascotti [141] cautioned that "although the salt dependence of $K_{\rm obs}$ is determined by the net charge of the oligopeptides, this is not the case for protein–nucleic acid interactions, since many nucleic acid binding proteins have a net negative charge at pH 7, and yet still bind strongly to the negatively charged nucleic acid". Extension of the Record treatment for oligolysine/DNA to protein/GAG (with the GAG polyelectrolyte now replacing DNA and the protein replacing oligolysine with a host–guest inversion) was initiated in 1991 by Olson et al. [52]. Citing Record et al. as "the theory of protein–polyelectrolyte interactions", they described the thrombin–heparin interaction as "an ionic exchange type process", with z representing "the number of ionic interactions that thrombin makes with heparin". Eq. (4) then appears as [142,143]:

$$\log K_{\rm d} = \log K_{\rm d}(nonionic) + z\psi \log \left[Na^+\right] \quad (5)$$

with the first term r.h.s. defined as "the nonionic contribution to the binding energy" characteristic of "a sequence-specific association…that derives from the complementarity of the interacting components".

On this basis, Thompson et al. concluded (from $K_{\rm d}$) that between two and three "net purely ionic interactions" are involved in the interaction of bFGF with low MW Hp, and (from the value of Kd extrapolated to 1 M salt) that only 33% of the binding energy is of electrostatic origin, while the remaining free energy due to H-bonding, van der Waals and hydrophobic interactions can be apportioned among a set of 5 or 6 amino acids, each one assigned an interaction free energy subdivided into electrostatic and non-electrostatic components. Citing Olson and Bjork [52], Friedrich et al. [143] used a similar approach to conclude that the interaction of Antithrombotic Protein C with Hp involved "4–6 ionic interactions" and that "34%" of the binding energy was nonionic. An interesting application of Eq. (5) appears in Ref. [55] mentioned above in which AT-binding was measured for a number of octasaccharides, all of which contained the "Fondaparinux" pentasaccharide. The highest affinity one "OCTA-4" bound AT 10-20x more strongly than Fondaparinux. Its trisaccharide "extension" provided an additional 5 negative charges (two carboxylates and three sulfates) on the nonreducing end of Fondaparinux, and provided AT-binding that was reduced by more than two orders of magnitude when the ionic strength was increased from 0.1 to 0.5 M, which might appear to be consistent with an electrostatic attraction between this extension and a locally positive AT domain. On the other hand, the application of Eq. (5) led to a very different conclusion. Since electrostatics are believed to be manifest in only the slope of the ionic strength dependence ($z\psi$), which was very similar for both Fondaparinux and OCTA 4, it was concluded that the much larger affinity for OCTA 4 could only arise from the nonionic term. Consequently the stronger binding by OCTA-4 was interpreted as meaning more "nonionic contacts" of an unspecificd nature. It is worth noting that while Olson et al, Friedrich et al. and others cite Manning [59] as one of the two theoretical foundations of this treatment, this reference has no expressions of the form of Eqs.(4) and (5), but rather of the form

$$\log K = const. + Z \log[\text{salt}] \quad (6)$$

wherein K includes some electrostatic contributions not dependent on salt concentration and the "const" includes both electrostatic and non-electrostatic effects (Manning, G.S., private communication).

It is instructive to compare Eqs. (3)–(5): the ionic strength enters Eq. (4) through entropy-driven ion pair formation, and allows the calculation of the number of ion pairs; and it designates as "nonionic" all deviations from a non-zero value of $K_{\rm obs}$ in the limit of $I = 1$ M. While Eq. (4) visualizes the complex as having short-range interactions only – ion pairs or "salt bridges" and "nonionic interactions" (generally undefined) – Eq. (3) allows for spatial separation of the two sets of charges. Ironically, the treatment, i.e. Eq. (3), applied by Beals et al. to a protein-receptor system (that would generally be considered "highly specific") is based on screened coulomb interactions and does not explicitly assign a meaning to other interactions of different nature. On the other hand, the treatments of the Hp-protein system, for which there is no a priori or experimental justification for either desolvation or for geometric charge complementarity (ion-pairing) of protein and heparin opposite charges, does not consider separation of those charges to allow for screening.

The extension of a theory – developed (and tested) for the binding of relatively simple oligocations to DNA – to the binding of proteins to GAGs is remarkable on several counts. First, an "ion-exchange" process implies considerable charge complementarity of the two species such that the two sets of charge can indeed "pair". Second, DNA with uniform charge spacing and well-defined geometry in the Record model is replaced by the semiflexible chain of Hp with highly irregular charge spacing, while the oligocation of the Record model is replaced by some undefined moiety of the protein. Third, all repulsive interactions between Hp and negative protein domains (representing the majority for most Hp binding proteins) are dismissed. The continuing appeal of this Procrustean approach to the far more complex GAG–protein world

might arise from a few aspects. Log–log plots representing Eq. (4) so often appear linear, particularly when restricted to less than an order of magnitude change in ionic strength. More fundamentally, it resolves a somewhat false dilemma arising from the misperception that electrostatics (being long-range) cannot be short range; and – since only short-range interactions can provide specificity – electrostatic interactions cannot be specific. The application of Eq. (5) admits to sizeable electrostatic contributions but offers, as mentioned above, an apartheid in which specific interactions (providing recognition) are separated from – and in a sense elevated above – non-specific interactions which provide a sort of directionless motive force for binding. As noted by Middaugh [39] "Much of this effort (on interaction between FGF and related GAGs and proteoglycans) has focused on the specificity of these interactions with little effort to address the opposite question, namely the absence of such specificity. As is often the case, the questions one asks very much determine the answers one receives."

A more nuanced and less contrived approach is reflected in statements about GAG–protein interactions in recent literature: (1) "there is an intermediate specificity based on a gradient of electrostatic interactions that are a function of relative charge densities in contrast to highly conformationally based structure specificity" in which "large variations in ligand (polyanion) structure do not necessarily dramatically lower the affinity of such contacts" [39]. (2) Since regulation of FGF signaling through interaction of HS with large scale reorganization of one or several components (e.g.FGF or FGFR) has a large thermodynamic penalty, "a mechanism involving little reorganization with some leeway in the stringency of fit could still produce reasonably high affinity" [2]. (3) Recognition can be primarily driven by electrostatic interactions, with contributions from both GAG conformational flexibilities and sulfate densities [133]. A dynamic nature of the bound state may be recognized, consistent with: (a) the need to preserve configurational entropy of native chain GAGs; (b) the presence of hydrated counterions compensating for the charges of the many non-ion-paired GAG and protein groups; and (c) the requirement that the conformation of GAGs bound to charge-anisotropic proteins should minimize repulsions as well as attractions (possibly explaining the separation of highly charge domains by more neutral ones within GAGs that bind to globally negative dimeric proteins with symmetric positive domains). Finally, as pointed out by Lindahl and Li in an excellent recent review on HS-protein interactions, "protein binding domains containing specific combinations of sulfate groups may be generated in regulated fashion, but without any requirement for synthesis of predetermined sequences. Whether such arrangement should be understood in terms of 'sequence specificity' appears essentially a semantic question". [1]. Views of specificity itself are changing in that it is increasingly recognized that most effects of HS are due to interactions that are "more-or-less electrostatic in nature" [ibid], with the role of ATIII–Hp essentially changing from model to anomaly.

7. Conclusions

Physicochemical studies show that GAGs exhibit the typical behavior of polyelectrolytes, i.e. statistical (or "random coil") chains the dimensions of which depend in the typical way on chain length and ionic strength. This conformity extends to many aspects of their interactions with proteins, including the ability to bind "on the wrong side of pI", the dependence of binding on ionic strength (including non-monotonic dependence when protein global charge is the same as GAG charge). Against the background of these generic properties, the exquisite regulation of structure (notably for HS) suggests that the thermodynamics and kinetics of protein binding show considerable sensitivity to the arrangement and sequencing of saccharides. Nevertheless, attempts to identify and explain "specificity" through directionally precise short-range bonds between GAG sulfates and protein amino acid side chains fail to consider polyelectrolyte chain entropy and intramolecular repulsion, and tend to confuse electrostatic interactions with ion-pair formation. The long-range aspect of these interactions assures that both repulsive and attractive effects are involved, suggesting a higher degree of electrostatic pattern recognition than commonly recognized. However, the extent to which structure regulation is equivalent to regulation of charge sequences is not fully understood. Generally speaking, current research appears to encompass more nuanced and interdisciplinary perspectives, as the field shifts away from the clinically relevant heparins, and towards more biologically significant GAGs (such as HS) where the Hp–AT paradigm is less imperative.

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