Identification of Structural Mechanisms that Modulate Glycosaminoglycan Affinity in Various Strains of Decorin Binding Protein A

by

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ABSTRACT

Glycosaminoglycans (GAGs) are a class of complex biomolecules comprised of linear, sulfated polysaccharides whose presence on cell surfaces and in the extracellular matrix involve them in many physiological phenomena as well as in interactions with pathogenic microbes. Decorin binding protein A (DBPA), a *Borrelia* surface lipoprotein involved in the infectivity of Lyme disease, is responsible for binding GAGs found on decorin, a small proteoglycan present in the extracellular matrix. Different DBPA strains have notable sequence heterogeneity that results in varying levels of GAG-binding affinity. In this dissertation, the structures and GAG-binding mechanisms for three strains of DBPA (B31 and N40 DBPAs from *B. burgdorferi* and PBr DBPA from *B. garinii*) are studied to determine why each strain has a different affinity for GAGs. These three strains have similar topologies consisting of five α-helices held together by a hydrophobic core as well as two long flexible segments: a linker between helices one and two and a C-terminal tail. This structural arrangement facilitates the formation of a basic pocket below the flexible linker which is the primary GAG-binding epitope. However, this GAG-binding site can be occluded by the flexible linker, which makes the linker a negative regulator of GAG-binding. ITC and NMR titrations provide $K_D$ values that show PBr DBPA binds GAGs with higher affinity than B31 and N40 DBPAs, while N40 binds with the lowest affinity of the three. Work in this thesis demonstrates that much of the discrepancies seen in GAG affinities of the three DBPAs can be explained by the amino acid composition and conformation of the linker. Mutagenesis studies show that B31 DBPA overcomes the pocket obstruction with the BXBB motif in its linker while PBr DBPA has a retracted linker that exposes the basic pocket as well as a
secondary GAG-binding site. N40 DBPA, however, does not have any evolutionary modifications to its structure to enhance GAG binding which explains its lower affinity for GAGs. GMSA and ELISA assays, along with NMR PRE experiments, confirm that structural changes in the linker do affect GAG-binding and, as a result, the linker is responsible for regulating GAG affinity.
DEDICATION

To Eric and my family
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CHAPTER 1

Introduction

1.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear, sulfated polysaccharides that are often found on the cell surface or in the extracellular matrix (ECM) of all mammals. Most GAGs are covalently linked to proteins forming entities known as proteoglycans, whose mass is more than 50% comprised of one or more GAG chains [1, 2]. GAGs play a role in many physiological phenomena, including cell adhesion, proliferation and migration, blood coagulation and immune responses to name a few [3]. Additionally, they are also often involved in pathogenic interactions with microbes. In particular, GAGs are the facilitators of the initial contact between the pathogen and the host cell. GAGs have been shown to be a target of numerous pathogens including, but not limited to, *Mycobacterium tuberculosis* (the causative agent of tuberculosis), herpes simplex virus, and *Plasmodium falciparum* (the causative agent of malaria) [3]. These pathogens have GAG-binding surface proteins that recognize and form weak interactions with the host cell’s GAGs. Interruption or manipulation of these interactions, which occur at the early stages of infection, could result in a major decrease of the pathogen’s invasive potential, making these strategies possible therapeutic approaches for treatment of infectious diseases [1, 3].

Structurally, all GAGs are comprised of repeating disaccharide units made up of one amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and one uronic sugar (glucuronic acid or iduronic acid) or galactose. Despite containing only two types of monosaccharides, GAGs are considered to be one of the most complex classes of biomolecules. This is primarily due to the number and variable positioning of sulfate
groups as well as the differences in epimerization (Table 1.1) [1, 4]. There are four structural families in which all GAGs fit: hyaluron (HA), heparin/heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS) and keratan sulfate (KS). However, the specific focus of these works is heparin, DS, and CS.


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<td>Hyaluron (HA)</td>
<td>~100-1000 kDa 379 Da</td>
<td>~260-2600</td>
<td>0</td>
<td>β 3 β 3 β 3</td>
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<tr>
<td>Heparin (HP)</td>
<td>~15 kDa 476.9 Da PD ~1.4</td>
<td>~30</td>
<td>~2.7</td>
<td>6S α 4 3S NS 4S α 4 2S α 4 3S NS</td>
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<tr>
<td>Heparan sulphate (HS)</td>
<td>~20 kDa 459.1 Da</td>
<td>~40</td>
<td>~1</td>
<td>α 4 α 4 α 4 α 4 NS</td>
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<td>Chondroitin sulphate - A (CSA)</td>
<td>~20 kDa 460 Da PD ~1.2</td>
<td>~40</td>
<td>~1</td>
<td>β 3 β 4 β 3</td>
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<td>Chondroitin sulphate-B (CSB or DS)</td>
<td>~30 kDa 459.1 Da</td>
<td>~60</td>
<td>~1</td>
<td>β 3 β 4 β 3</td>
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<td>Chondroitin sulphate-C (GSC)</td>
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<td>~40</td>
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<td>~20 kDa 530 Da</td>
<td>~40</td>
<td>~2</td>
<td>6S α 4 3S NS 4S α 4 2S α 4 3S NS</td>
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<td>Chondroitin sulphate-E (CSE)</td>
<td>~20 kDa 530 Da</td>
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<td>Keratan sulphate-I (KS I)</td>
<td>~14 kDa 500 Da</td>
<td>~35 ~ 10</td>
<td>~1.5</td>
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<td>Keratan sulphate-II (KS II)</td>
<td>~5 kDa 500 Da</td>
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<td>β 4 β 4 β 4</td>
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*Gluconic acid (GlcA), Glucosamine (GlcN), N-Acetylglucosamine (GlcNAc), Iduronic acid (IdoA), Galactose (Gal), N-Acetylgalactosamine (GalNAc)*

Structure of the major disaccharide units are represented based on the consortium for functional glycobiology nomenclature. The abbreviations 2S, 4S or 6S indicate 2-O, 4-O or 6-O-sulpho, respectively. Mw, molecular weight.

Heparin is the most complex of all the GAGs because it has the highest level of sulfation, giving it a high charge density. As seen in Figure 1.1, it is comprised of iduronic acid (IdoA) linked α1-4 to N-acetyl (or N-sulfo) glucosamine (GlcNAc or GlcNS). CS and DS both have a much lower level of sulfation than heparin and are more
structurally similar to each other than to heparin. The major repeating unit for CS is

\[ \text{glucuronic acid (GlcA)} \text{ linked } \beta 1-3 \text{ to N-acetylgalactosamine (GalNAc)} \text{ which is linked } \beta 1-4 \text{ to the next unit while DS is the same as CS but contains IdoA rather than GlcA} \]

(Figure 1.1) [1, 2, 5].

**Figure 1.1.** Chemical structures of representative disaccharide units found in HS/heparin, CS, and DS. Sulfate groups are colored red and alternate sulfation positions are colored green.

Biosynthesis of GAGs takes place post-translationally in both the ER and the Golgi apparatus. Because these GAGs are modified without any template, the sulfation and epimerization reactions may not go to completion and the extent of these modifications is highly dependent on enzyme levels produced by the cell, leading to a heterogeneous mixture of GAG chains *in vivo*. The majority of GAGs can be found on the cell surface and in the ECM [1, 2, 4]. Specifically, heparin is stored in mast cells so it can be found in the blood (and blood vessel connective tissue), muscle, liver and lungs, while CS and DS are more prominent in bone, tendon, cartilage, blood vessels, and nerve tissue of most mammals [1, 5]. Due to the many similarities of these GAGs as well as their diverse tissue locations, heparin, DS and CS have a broad range of GAG-binding proteins with which they can interact.

1.2 Background on Lyme Disease

Lyme disease, also known as Lyme borreliosis, is the most common vector-borne disease in the United States but can also be found worldwide [6, 7]. There are three stages
to Lyme disease: primary (early localized infection), secondary (early disseminated infection), and tertiary (late persistent infection) [8]. Common symptoms of this disease range from erythema migrans (bull’s-eye rash) and flu-like symptoms (primary) to acute neurological and cardiovascular problems in the secondary stage and then on to chronic neurological, cardiovascular and skin disorders as well as arthritis (tertiary). Lyme disease can be contracted after being bitten by a nymphal *Ixodes* tick infected with *Borrelia burgdorferi* (North America, specifically northeastern U.S.), *B. garinii* (Europe and Asia) or *B. afzelii* (Europe and Asia) [7]. Specifically, *B. burgdorferi* predominantly results in arthritis while *B. garinii* and *B. afzelii* cause neurological disorders and skin problems, respectively [8]. The disease can also be spread by the saliva of the adult tick as well but as they are larger and easier to find, they do not remain attached to the host for an extended period of time. The nymph normally has to remain attached to the host for 1 to 2 days to allow the bacteria to be transmitted [8].

*Borrelia* is a spirochete bacterium with GAG-binding surface lipoproteins which relies on interactions with GAGs to allow it to initially disseminate through blood vessels and colonize in the ECM of the joints [7-9]. The *Borrelia* bacterium is often found bound to decorin, a small DS proteoglycan found in the ECM that binds to collagen, thus aligning the bacterium in the ECM and promoting colonization [7, 10]. The spread of the bacterium to the ECM from the blood is required for chronic infection and renders antibiotics less effective [8, 11, 12]. Once colonization begins, the bacterium requires nutrients from the host to survive and grow but, surprisingly, *Borrelia* has been shown to not require iron for growth in vitro which may aid the bacterium in evading the host’s immune response [9]. In addition to the lack of efficacy in treating Lyme disease through
antibiotics or the immune system, an effective vaccination against Lyme disease has proven to be difficult to produce due to the high genetic variability of the strains of *Borrelia* [8, 13].

1.3 Decorin Binding Protein A

A potential therapeutic target for Lyme disease is decorin binding protein (DBP). DBPs are one of *B. burgdorferi*’s surface lipoproteins expressed during the early host infection stage and are the primary adhesins for decorin [10, 14]. DBPs are GAG-binding proteins so they interact strongly with the DS GAG chains found on decorin; although, there is no current evidence to disprove that the core protein may also be involved in binding [10, 14-16]. However, DBPs can also form attachments to other proteoglycans that contain other GAG chains than DS [10, 14, 17, 18]. Therefore, DBPs have been shown *in vivo* and *in vitro* to bind heparin and HS but not to CS [5, 15-17, 19, 20]. In fact, DBPs’ affinity for heparin is higher than that of DS [20, 21].

There are two homologous forms of DBP (DBPA and DBPB) in *B. burgdorferi*. Both isoforms of DBP, as well as decorin, are required for effectiveness of the infection process in the early stages as deletion of the DBPA/B gene reduces virulence by at least three orders of magnitude [22-24]. However, DBPA only shares approximately 40% sequence identity with DBPB. Furthermore, the sequences found in the strains of DBPB are highly conserved while the sequences in DBPA are much more heterogeneous among the different strains with sequence identities that range from 60% to 99.5% [19, 25]. Specifically, the commonly studied strains from *B. burgdorferi* N40 and B356 are 99% homologous but are only ~75% homologous with strains B31 and 297 which share 90% sequence identity between themselves [19]. However, PBr DBPA from *B. garinii* and
VS461 from *B. afzelii* have ~70% and ~60% sequence homology with the *B. burgdorferi* strains, respectively. An in-depth study conducted by Benoit et al. showed that PBr DBPA bound GAGs with the greatest affinity while N40 DBPA had one of the lowest GAG-binding affinities [19, 26]. As expected, the strains that had greater than 90% sequence homology also had similar levels of GAG-binding affinity (Figure 1.2).

![Graph showing DS and decorin binding levels](image)

**Figure 1.2.** DS and decorin binding levels vary among the commonly studied DBPA alleles [19]. (Reprinted with permission from Benoit V. M. et al. (2011) *Infect. Immun.*, 79(9), 3501-3509. © 2011 American Society for Microbiology. See Appendix B.)

Additionally, this variation in sequence found in DBPA strains is also sufficient to induce differences in tissue preference [15, 19, 26]. For example, Leong and co-workers demonstrated that the bacteria expressing PBr DBPA from *B. garinii*, which binds intact DS with a $K_D$ of ~0.21 µM, preferentially localized to the heart; whereas, the same bacteria expressing N40 DBPA, which bound DS with much lower affinity ($K_D$ ~3.10 µM) and produced better localization in the knee [26]. While both isoforms of DBP are necessary for virulence and tissue colonization, a study by Shi et al. showed that DBPA and DBPB do not contribute equally to the infectivity of *Borrelia* but that DBPA deficiency may result in a greater decrease in infectivity than DBPB deficiency, making DBPA the more interesting therapeutic target [23, 24, 27, 28].
Surprisingly, these strains of DBPA share similar structures despite the differences in their sequences. As can be seen by the first solution structure determined by NMR [21], B31 DBPA is a helical protein consisting of five α-helices held together in a bundle by a strong hydrophobic core (Figure 1.3). It also has a flexible linker present between helices one and two as well as a flexible C-terminal tail, the dynamics of which were confirmed through NMR relaxation studies [21]. This structure was later confirmed by an X-ray crystal study [29]. Electrostatic potential mapping revealed a basic pocket below the flexible linker and bounded by helices two, four and five that is the primary GAG-binding epitope.

![Figure 1.3. Ribbon depiction of B31 DBPA with the sidechains of the hydrophobic core in yellow. Helix one (residues 30-56) is green, helix two (residues 75-104) is blue, helix three (residues 109-129) is red, helix four (residues 133-145) is cyan, and helix five (residues 149-178) is purple. The schematic topology of the protein is at the bottom left [21]. (Adapted with permission from Wang, X. (2012) Biochemistry, 42, 8353-83625. © 2012 American Chemical Society. See Appendix C.)](image)

In fact, three basic residues deemed by previous studies to be crucial for GAG binding can be found in this basic pocket. These residues, K82, K163, and K170, were first
identified by Hook et al. as important for GAG binding through sequence alignment and peptide screening [30]. K82 is found on helix two while K163 and K170 both reside on helix five, putting all three residues in the pocket. In addition to these lysines, the C-terminus of VS461 (comparable to the last thirteen residues of B31 DBPA) was also discovered to be important for GAG-binding [19]. Although, these binding motifs are conserved among many of the strains of DBPA making them important for binding, their presence is not a good predictor of GAG-binding affinities. In this work, the structures of two strains of DBPA (N40 from B. burgdorferi and PBr from B. garinii) were determined with solution NMR and compared to the known solution structure of B31 DBPA from B. burgdorferi to identify the unique characteristics that contribute to the varying levels of GAG-binding affinity. Once identified, mutagenesis studies were conducted in conjunction with gel mobility shift assays, ELISA assays, NMR-monitored titrations, isothermal titration calorimetry and paramagnetic relaxation enhancement to confirm the effect of each motif on GAG binding. Results of these studies showed that the flexible linker between helices one and two acts as a regulator for GAG-binding affinity and evolutionary changes to this linker can not only affect GAG affinity but may also be responsible for the tissue specificity experienced by each strain of DBPA.

1.4 Techniques for Characterizing Protein-GAG Interactions

1.4.1 Gel Mobility Shift Assay

A gel mobility shift assay (GMSA) is an electrophoretic technique typically used to study the affinity of protein-DNA or protein-RNA interactions. Rosenberg and co-workers were the first to adopt this technique to qualitatively determine the affinity of protein-GAG interactions [31]. GAG fragments of known size are labeled with 2-
aminoacridone (2-AMAC), a fluorescent aromatic tag with a primary amine that reacts with the aldehyde at the reducing end of the GAG as seen in Figure 1.4 [32].

![Structure of 2-AMAC](image)

**Figure 1.4.** Structure of 2-AMAC used for the preparation of the fluorescently labeled GAG [33].

Once the fluorescent GAG is incubated with various concentrations of protein, the mixture is run on an agarose gel for a short time and the result is visualized with UV [31, 32]. Conceptually, if the protein is bound strongly to the GAG, then the complex, due to its larger size and change in overall charge, will move much slower than the free, unbound GAGs resulting in a complex that migrates slower in the electrophoresis and is ‘shifted’ up on the gel. Therefore, the strength various protein-GAG interactions can be qualitatively determined using GMSA.

1.4.2 ELISA

ELISA (enzyme-linked immunosorbent assay) is a quantitative test that detects protein-GAG interactions colorimetrically. The challenge in GAG-binding ELISA is finding a reliable method for GAG immobilization. In some of our assays, the intact GAGs are biotinylated and immobilized on a streptavidin-coated microplate through the strong biotin-avidin interaction (Figure 1.5).
Figure 1.5. Schematic of ELISA utilizing biotinylated GAGs immobilized on a streptavidin-coated microplate to bind His-tagged DBPA.

The coated wells are then incubated with tagged GAG-binding protein of increasing concentrations to test their binding affinity for the GAG. To quantitate the amount of bound protein, a primary antibody, which is often conjugated to the enzyme horseradish peroxidase (HRP) and specific to the protein tag, is then added to the wells after removing unbound proteins. Addition of substrate for the enzyme results in a color change as the reaction is catalyzed (Figure 1.5). The more protein that is bound, the darker the color change. In most of the assays presented in this thesis, DBPA was expressed as a fusion protein with N-terminal His-tagged ubiquitin tag and anti-His HRP was used as the primary antibody. The HRP reaction produces a bright blue color change upon the addition of the TMB (3,3’,5,5’-tetramethylbenzidine) substrate.
1.4.3 NMR Titrations of Protein with GAGs

NMR characterization of protein-GAG interactions are primarily through spectra of $^{15}$N isotopic labeled proteins. Once labeled, $^1$H—$^{15}$N HSQC spectra are collected of the protein at increasing concentrations of a GAG ligand. Ideally, the titration should continue until the protein has been saturated but for a weak binding protein, this endpoint may be difficult to detect. Large chemical shift changes of the backbone amide nitrogen and hydrogen can be used to obtain the dissociation constant, or $K_D$, of the protein [34]. However, chemical shifts are sensitive to changes in other properties like pH and temperature, resulting in small oscillations in peak positions. As a result, the $K_D$ value extracted is more reliable if signals experiencing large chemical shift changes can be found. The chemical shift changes in the $^1$H and $^{15}$N dimensions are combined to produce one value ($\delta$) for each titration point using with Equation 1.1 where $\delta_H$ and $\delta_N$ represent the chemical shifts for $^1$H and $^{15}$N, respectively [35].

$$\delta = [\Delta\delta_H^2 + (1.7 \times \Delta\delta_N)^2]^{1/2} \quad (1.1)$$

For systems in which each molecule of protein only binds a single molecule of ligand, the change in chemical shifts of protein atoms can be described by Equation 1.2 where $\Delta\delta$ is the observed change in chemical shift, $\Delta\delta_{\text{max}}$ is the maximum change in chemical shift at saturation, $[P]_i$ and $[L]_i$ are the total protein and ligand concentrations, respectively [36].

$$\Delta\delta = \Delta\delta_{\text{max}} \frac{(K_D + [P]_i + [L]_i) - \sqrt{(K_D + [P]_i + [L]_i)^2 - 4([P]_i[L]_i)}}{2[P]_i} \quad (1.2)$$

Fitting the chemical shift changes at different ligand concentrations to this equation allows the $K_D$ of a particular amino acid to be determined.
1.4.4 Isothermal Titration Calorimetry

The $K_D$ of a protein-GAG interaction can also be determined quantitatively using isothermal titration calorimetry (ITC). Conceptually, ITC works by measuring the heat released or absorbed during the binding of the GAG to the protein. This heat transfer ($\Delta H$ or enthalpy) can also be used to derive $K_D$, which can then be used to calculate $\Delta G$ (Gibb’s free energy) and $\Delta S$ (entropy) of the protein-GAG interaction; this is measured directly and without labels on the protein or the GAG. The heat released or absorbed is measured by a sensitive calorimeter while the GAG is gradually being added into the sample cell containing the GAG-binding protein. The maximum change in heat observed in the raw data gives the value for $\Delta H$. Then, the isotherm created from integration of the raw data is fitted according to a binding model, for example a one-to-one ligand-protein binding ($X$—$M$) reaction where $X + M \leftrightarrow MX$. For this binding, the change in heat of the system normalized with respect to the moles of ligand added per injection ($\frac{dQ}{d[X]}$) is related to the ratio of ligand-to-protein concentration ($X_R = \frac{[X]}{[M]}$) at any point in the titration with Equation 1.3

$$\frac{dQ}{d[X]}_t = \Delta HV_0 \left[ \frac{1}{2} + \frac{1 - X_R^{-r}}{2 \sqrt{(1 + X_R + r)^2 - 4X_R}} \right]$$

(1.3)

where $1/r = c$ and $V_0$ is the volume of the calorimeter cell [37]. The constant, $c$, which determines the shape of the isotherm, can give the $K_D$ of the interaction as seen in Equation 1.4 where $n$ is the stoichiometry of the interaction and $[P]_t$ is the total protein concentration [37].

$$c = \frac{n[P]_t}{K_D}$$

(1.4)
Using the $K_D$ value calculated in Equation 1.4, the $\Delta G$ can be calculated from the equation,

$$\Delta G = RT \ln(K_D)$$  \hspace{1cm} (1.5)

where R is the gas constant and T is the experimental temperature. Once the $\Delta G$ is determined, the entropy of binding ($\Delta S$) at the experimental temperature can be calculated with Equation 1.6 [38].

$$\Delta G = \Delta H - T\Delta S$$ \hspace{1cm} (1.6)

ITC allows for the simultaneous determination of the thermodynamic parameters $\Delta H$, $K_D$, $\Delta G$, and T$\Delta S$ which can give important quantitative information regarding GAG-protein interactions [37, 38].

1.4.5 Paramagnetic Relaxation Enhancement

Paramagnetic relaxation enhancement (PRE) is a result of the magnetic dipolar interactions between a nucleus and an unpaired electron that manifests itself as an increase in the relaxation rates of both the longitudinal and transverse magnetization. In particular, the paramagnetic enhancement to the rate of proton transverse relaxation ($R_{2,\text{PRE}}$) produced by a single electron can be described by Equation 1.7, where $r$ is the distance between the paramagnetic center and the proton, $\gamma_H$ is the gyromagnetic ratio of the proton, $g_s$ is the electron g factor for the unpaired electron, $\omega_H/2\pi$ is the resonance frequency of the proton, and $\tau_c$ is the combined rotational correlation time for the entire system [39].

$$R_{2,\text{PRE}} = \frac{1}{15} \left( \frac{\mu_0}{4\pi} \right)^2 \gamma_H^2 g_s^2 \mu_B^2 S(S+1) \left( \frac{4\tau_c}{r^6} + \frac{3\tau_c}{1 + \omega_H^2 \tau_c^2} \right)$$ \hspace{1cm} (1.7)

Similar to the nuclear Overhauser effect (NOE), the magnitude of PRE is distance-dependent as it is proportional to $r^{-6}$. However, due to the large magnetic moment of the
electron, the PRE effect gives information over much longer distances than NOE. GAG fragments labeled with 4-amino-TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), a stable nitrooxide radical, has been used to detect GAG binding with high sensitivity in this thesis. The PRE rate can be quantitatively determined by measuring the difference in transverse relaxation rates of protein atoms when bound to either the oxidized TEMPO or the reduced TEMPO. As the TEMPO tag is attached to the reducing end of the GAG, this PRE effect can also give information on the orientation of the GAG relative to the protein.

Another application of PRE to the study of protein-GAG interactions is to probe the basic areas on the surface of the protein with an acidic paramagnetic ligand and then competing it off with the protein’s natural GAG ligand. Binding of the GAG to the protein will displace the paramagnetic ligand, resulting in the return of the NMR signal. This application is useful in probing potential GAG-binding epitopes. The degree of protection from paramagnetic compounds offered by the GAG ligand can be quantified by the change in PRE experienced by the atom in the presence and absence of GAG ligands. The $R_{2\text{PRE}}$ can also be estimated from HSQC's of the protein bound to the diamagnetic and paramagnetic forms of the ligand using Equation 1.8 where $I_{\text{para}}(0)$ and $I_{\text{dia}}(0)$ are peak intensities of the oxidized and reduced states, respectively, $R_{2\text{dia}}$ is the transverse relaxation rate in the reduced state (calculated from the line width at half height), and $t$ is the overall time of $^1$H transverse relaxation during the pulse sequence [40, 41].

$$\frac{I_{\text{para}}}{I_{\text{dia}}} = \frac{R_{2\text{dia}}\exp(-R_{2\text{para}}t)}{R_{2\text{dia}} + R_{2\text{para}}}$$

(1.8)
Because the magnitude of PRE is dependent on gyromagnetic ratios, measuring the $^1\text{H}$—$\Gamma_2$ (transverse PRE) gives the most precise and accurate measurement of PRE due to the lack of susceptibility to internal motions and cross-relaxation as well as the high sensitivity of the proton as a probe. In particular, the transverse relaxation rates of each backbone amide hydrogen can be most conveniently measured to determine which residues experienced the largest effect thus indicating which residues are available to bind GAGs.
CHAPTER 2

Novel Heparin-Binding Motif in Decorin Binding Protein A from Strain B31 of Borrelia burgdorferi Explains Higher Binding Affinity


2.1 Introduction

Both in vivo and in vitro studies have conclusively shown that the GAG segment of decorin is the most biologically relevant binding site for DBPs [14-16]. DBPA, in particular, is known to have affinity for a variety of GAGs. In fact, in vitro studies have revealed that DBPA binds heparin with greater affinity than other GAGs such as DS or CS [15-17, 19-21]. However, DBPA’s binding affinity for GAGs seems to differ wildly among different Borrelia strains. Because of DBPA’s role as an ECM adhesin, its GAG-binding affinity may be a crucial determinant in Borrelia infectivity, making understanding the molecular mechanism underlying it interactions with GAGs a priority. Furthermore, the void in our knowledge of GAG-protein interactions in general means DBPA’s sequence-dependent GAG affinity is an excellent opportunity to investigate principles governing GAG-protein interactions. However, there is yet no molecular explanation for the large deviations observed in GAG binding affinities of DBPAs from different strains of B. burgdorferi.

Attempts to identify GAG-binding epitopes of DBPA started soon after the protein’s discovery. Using sequence alignment and a peptide screening approach, Hook and co-workers first identified residues K82, K163, and K170 as being critical to the decorin binding ability of DBPA [30]. More recently, Benoit et al. serendipitously
discovered that the absence of the C-terminus of DBPA from strain VS461 (last 11-13 residues of DBPA) abrogated the protein’s ability to bind GAGs [19]. Unfortunately, because these motifs are shared among most strains of *B. burgdorferi*, they do not reveal the structural rationale behind differing GAG affinities between strains B31 and N40. However, the recently determined structure of B31 DBPA does give one clue about the reason behind B31’s enhanced GAG affinity: according to the structure, the proposed GAG-binding epitope on B31 is a basic pocket consisting of helices and two dynamic segments of DBPA. The previously identified GAG-binding motifs mentioned above are all found in the pocket (Figure 2.1) [21]. Most intriguingly, one of the flexible segments, a linker between helices 1 and 2 covering the pocket (residues 58-72), contains a BXBB motif (B represents a basic amino acid) not seen in many versions of DBPA, including those from strains N40 and B356.
Figure 2.1. Ribbon depiction of wild-type B31 DBPA with the sidechains of the mutated lysine residues colored blue.

Guided by the structure for B31, we are now able to propose a hypothesis explaining the differences in GAG affinities of DBPAs from strains B31 and N40. In particular, we believe the linker BXBB motif may be significantly important in promoting DBPA-GAG interactions. In this work, we report the biophysical and structural characterization of the interactions between size-defined GAG fragments and known GAG-binding motifs on B31 DBPA. Our choice of GAG ligands is size-defined low-molecular weight heparin. This decision is prompted by the fact that DBPA has a very low affinity for low-molecular weight DS \textit{in vitro}, which has made accurate characterizations of DBPA’s GAG affinity impractical [21]. Heparan sulfates, a form of
heparin that resides on the epithelial cell surface, are known to act as receptors for \textit{B. burgdorferi} \cite{17}, and given the promiscuity of most GAG-binding proteins, epitopes identified with heparin should be applicable to the binding of other types of GAGs. Through the use of fluorescence-assisted GMSAs, we showed the BXBB cluster in the linker is vital to the GAG affinity of B31 DBPA, such that B31 DBPA’s affinity for both low-molecular weight heparin and DS decreases significantly in its absence. The free energy contribution of each motif to GAG binding was also quantitatively characterized using NMR and ITC, and the result correlated well with those observed in gel mobility shift assays. In particular, the linker BXBB motif and the three lysine residues on the helices contributed most to the free energy of binding, and the C-terminal residues contributed much less. The direct interaction of the linker residues with GAGs was confirmed through a NMR PRE study using a novel TEMPO-tagged paramagnetic heparin ligand. The results unambiguously proved that the linker bound not only heparin but also the heparin fragment oriented specifically with the reducing end of the fragment close to the linker. Using observations in this study, a rationale for the higher GAG affinity seen in B31 DBPA can now be offered along with a structural model for the interactions between GAG fragments and B31.

2.2 Experimental Procedures

2.2.1 Expression and Purification of B31 Variants

The open reading frame for the wild-type (WT) mature B31 DBPA (residues 24-191) was synthesized by Genscript Inc. (Piscataway, NJ) and cloned into the pHUE vector that incorporates His-tagged ubiquitin at the N-terminus of B31 to give a fusion protein \cite{42}. Mutagenic primers were designed for four B31 mutants: $^{64}\text{KDKK}^{67}$ to
The forward primers for the mutants were as follows:

($^{64}$SDSS$^{67}$) 5’-GCGGTTAACTTCGATGCCTTCAGCGATAGCAGCACCGGCAGTTGTGAGCGAAAATCCG-3’, ($^{176}$SSS$^{178}$) 5’-CAAAAAACTACTGCACGCTGAGCAGCAGCGAAATAGCACCTTC-3’, ($^{187}$SCS$^{189}$) 5’-GCACCTTCACGGATGAAAGCTGTAGCAACAATTGAAAGCTTAG-3’, (K82S) 5’-CCGTTTATCCTGGAAGCCAGCGTGCGTGCAACCACG-3’, and (K170S) 5’-CTGCAGCGCTGCATACCACTACTGCACGCTG-3’. Mutagenesis was conducted with the Agilent Quikchange site-directed mutagenesis kit by following the manufacturer’s instructions, and the incorporation of the mutations was confirmed through sequencing.

To express the protein, the plasmid was transformed into *Escherichia coli* (E. coli) BL21(DE3), and the bacteria were grown at 37°C in M9 medium to an OD$_{600}$ of 0.5. The M9 medium was supplemented with $^{15}$NH$_4$Cl and/or $[^{13}$C]-glucose depending on the desired isotopic labeling scheme. The bacteria were induced with 0.5 mM IPTG and incubated overnight at 30°C. The cells were harvested via centrifugation, and the resuspended cell pellet was incubated with 1 mg/mL lysozyme and then sonicated to lyse the cells. The fusion protein in the supernatant was obtained via Ni affinity chromatography using a 1 mL HisTrap column (GE Life Sciences). The fusion protein was eluted off the column using an imidazole gradient from 35 to 500 mM at a flow rate of 1 mL/min. The fusion protein was exchanged into 25 mM Tris (pH 8.0) and 100 mM NaCl and digested with USP2 and 1 mM DTT overnight at room temperature [42]. The
cleaved DBPA was purified using a 1 mL HisTrap column. The cleaved DBPA was found in the flow-through, which was collected and concentrated. Figure 2.2 shows the SDS-PAGE analysis of the sample during each stage of purification.

Figure 2.2. SDS-PAGE gel showing the purification of B31 DBPA. Lanes 1 and 3 contain the before induction samples while lanes 2 and 4 show the production of Ubi-B31 after induction. Lane 5 shows the flow-through from the first Ni²⁺ column. Purification with a second Ni²⁺ column after digestion resulted in the purified protein (Lane 6) and elution fractions containing undigested B31 and ubiquitin (Lanes 7 and 8).

2.2.2 Production of Heparin and TEMPO-Labeled Heparin Fragments

Heparin and DS purchased from Sigma-Aldrich were first dialyzed and lyophilized to remove excess salt. Porcine mucosa heparin was digested with 0.5 IU of heparinase I (IBEX Inc.), and DS was digested with Chondroitinase ABC (Sigma-Aldrich) until the depolymerization was 30% complete to give short fragments [43]. The fragments were separated using a 2.5 cm × 175 cm size exclusion chromatography
column (Bio-Rad Biogel P10) with a flow rate of 0.2 mL/min. The fractions containing the same size were pooled, desalted, and lyophilized. No further steps were taken to separate fragments bearing different sulfation patterns. Disaccharide analysis of the fragments used showed that heparin fragments contained ~45% disulfated disaccharides and ~40% trisulfated disaccharides and DS contained mostly monosulfated disaccharides. For the PRE study, the reducing end of heparin hexasaccharide (dp6) fragments was modified using a nitroxide radical, 4-amino-TEMPO, through reductive amination (Figure 2.3).

![Figure 2.3](image)

**Figure 2.3.** Reducing end TEMPO-labeling of heparin fragments through reductive amination. Specifically, 300 µM TEMPO was incubated with 1 mg of the heparin fragment and 25 mM NaCNBH₃ at 65°C in water for 3 hours. The mixture was then desalted, and GAG fragments were isolated using SAX-HPLC.

2.2.3 Gel Mobility Shift Assays for WT B31 and B31 Mutants

Heparin decasaccharides (dp10), heparin dp6, and DS hexasaccharides were fluorescently labeled with 0.1 M 2-AMAC [32]. Briefly, 5 µL of 20 mg/mL GAG fragment was mixed with 40 µL of 0.1 M 2-AMAC that had been dissolved in 85% Me₂SO and 15% glacial acetic acid. This mixture was incubated at 37°C overnight and then precipitated by adding 9 volumes of ethanol and incubating the mixture at -20°C for 15 minutes. After centrifugation, the pellet was washed with an additional 9 volumes of
ethanol, and the resulting pellet was suspended in 50 Mm sodium phosphate (pH 6.5) and 150 mM NaCl.

To perform the gel mobility shift assays, 1 µg of the fluorescently labeled heparin fragment was mixed with 0.5, 1, or 2 molar equivalents of WT B31 or B31 mutants in 50 mM sodium phosphate (pH 6.5) and 150 mM NaCl buffer. For the DS dp6 GMSA, the fluorescently labeled fragments were mixed with the protein at a DS-to-protein ratio of 3, and in 50 mM acetate (pH 5) and 150 mM NaCl buffer. The control is the same mixture but with an equal volume of the same buffer without the protein. The reaction mixtures were incubated at room temperature for 30 minutes and were run at 120 V for 15 to 25 minutes in a 1% agarose gel immersed in the same buffer as the buffer used for incubation. A UV panel was used to visualize the shifts [44].

2.2.4 Titrations of WT and Mutant B31 Using Heparin dp6

K_D values of the interaction between heparin dp6 and B31 were estimated using NMR-monitored titration. Specifically, 2.10 mM heparin dp6 was added to 400 µL of 150 µM DBPA in 300 µM aliquots. This was done for B31 WT, B31 64SDSS 67, B31 187SCS 189, and B31 K82,170S. For B31 176SSS 178, 3.60 mM heparin dp6 was added to 150 µM DBPA in 600 µM aliquots. The pH of all the protein samples was lowered from 6.5 to 5.0. A 1H—15N HSQC spectrum was collected at each titration point. The chemical shift changes in the 1H and 15N dimensions were normalized into one chemical shift value [35]. The normalized chemical shift was calculated using Equation 1.1. The K_D of binding was extracted using the fitting feature in xcrvfit (http://www.bionmr.ualberta.ca/bds/software/xcrvfit/) to plot the normalized chemical
shift against the ratio of ligand to protein. Data for the titrations were collected on a Bruker Ultra-Shield 600 MHz and 850 MHz spectrometers.

2.2.5 Isothermal Titration Calorimetry of DBPA-Heparin Interactions

ITC of WT B31 DBPA and B31 mutants with heparin dp6 was performed on a Microcal ITC-200 calorimeter. Samples consisting of 300 µL of 200 µM DBPA were titrated with aliquots of 10 mM heparin dp6 stock solution at 25°C. Buffer containing no protein was used as a reference. Each titration was repeated three times, and average values of the dissociation constant and enthalpy change are reported. (ITC titrations were performed by Dr. Andrey Bobokov of Sanford-Burnham Medical Research Institute.)

2.2.6 Acquisition and Analysis of Backbone Dynamics Data

All NMR samples consisted of 400 µL of 150-200 µM 15N-labeled B31 WT DBPA in 50 mM NaH₂PO₄ (pH 6.5) and 150 mM NaCl buffer. NMR data for relaxation spectra were collected on Bruker Ultra-Shield 600 and Varian Inova 800 MHz spectrometers. Paramagnetic relaxation enhancement (PRE) from TEMPO-labeled heparin fragments was quantified by measuring ¹H T₂ values of the backbone amide protons according to the method of Iwahara et al. [45] before and after the radical on the heparin fragment was reduced with ascorbic acid. Relaxation delays of 1, 4.5, 8, 11.5, 15, and 18.5 ms were used. The difference in the ¹H T₂ before and after reduction of the radical is the relaxation contribution from the TEMPO radical.

T₁, T₂, and steady state heteronuclear nuclear Overhauser effect (NOE) experiments were conducted for WT B31 in the presence and absence of 24 molar equivalents of heparin dp6. The relaxation delays for the T₁ experiments were 0.1, 0.3, 0.5, 0.7, 0.9, and 1.3 s. The relaxation delays for the T₂ experiments were 10, 30, 50, and
70 ms. Steady state heteronuclear NOEs were derived from peak intensity ratios of spectra collected with and without proton saturation for 3 s. The data were processed with NMRPipe [46] and analyzed using NMRView [47]. The order parameter, $S^2$, was extracted using the model-free approach with relax, model-free software [48]. The protein is assumed to undergo isotropic global rotational diffusion, and its global rotational correlation time, $\tau_m$, was estimated as the average rotational correlation times of all residues in the structured region. The residue-specific correlation times were calculated according to equation 9 of Kay et al. [49], taking into consideration only contributions from $J(0)$ and $J(\omega_N)$. In particular, Equation 2.1 was used, where $\nu_N$ is the resonance frequency of $^{15}$N in Hz.

$$\tau_c = \frac{1}{4\pi\nu_N} \sqrt{\frac{T_1}{T_2} - 7}$$

(2.1)

DBPA’s internal motions were characterized through the fitting parameters of $S^2$ (magnitude of internal motion), $\tau_c$ (internal motion correlation time), and $R_{ex}$ (contribution of conformational exchange to transverse relaxation) for each backbone amide nitrogen atom.

2.3 Results

2.3.1 Interaction of B31 Mutants with GAGs

To assess the importance of known B31 DBPA GAG-binding motifs to GAG interactions, we created four B31 mutants each lacking a basic amino acid cluster from one of the proposed GAG-binding motifs. The linker mutant, $^{64}$SDSS$^{67}$, contains the mutations of residues $^{64}$KDKK$^{67}$ in the linker to $^{64}$SDSS$^{67}$. The $^{176}$SSS$^{178}$ mutant contains the mutations of C-terminal residues $^{176}$KKK$^{178}$ to $^{176}$SSS$^{178}$. Similarly, the $^{187}$SCS$^{189}$ mutant contains the mutations of C-terminal residues $^{187}$KCK$^{189}$ to $^{187}$SCS$^{189}$. Finally, the
K82,170S mutant contains the mutations of two helical residues first identified as being part of the GAG-binding epitope [30, 50]. Because native polymers of GAGs induce only signal disappearance when added to samples of DBPA [21], uniform-sized fragments of GAGs were used to evaluate mutagenesis-related changes in DBPA’s GAG affinity. Furthermore, heparin dp6 was chosen as the primary ligand. The choice is a consequence of DBPA’s relatively strong affinity for heparin. Similarly sized fragments of both DS and CS, the GAG types found on decorin, do not interact strongly with DBPA even under the most optimized conditions [15, 19, 21].

A qualitative characterization of B31-heparin interactions was first conducted with a gel mobility shift assay and fluorescently labeled heparin dp10 fragments [44]. The gel mobility shift assay is based on the principle that the migration of the heparin fragments is greatly impeded when proteins are bound. The effect is a combination of an increase in the apparent size of the complex and a reduction in the net charge of the complex. Using such an assay, we measured the GAG affinity of wild-type B31 as well as the linker mutant $^{64}\text{SDSS}^{67}$, C-terminal mutants $^{176}\text{SSS}^{178}$ and $^{187}\text{SCS}^{189}$, and helical mutant K82,170S. The results are shown in Figure 2.4.
Figure 2.4. Gel mobility shift assay of heparin dp10 in the presence of increasing concentrations of (A) B31 WT, 64SDSS67, 176SSS178, and 187SCS189 and of (B) B31 WT, 64SDSS67, and K82,170S.

The assay clearly demonstrated that WT B31 is capable of inducing shifts in a significant proportion of the heparin fragments. However, the four B31 mutants did not have a similarly strong effect on heparin fragment migration (Figure 2.4). 64SDSS67 and K82,170S mutants showed no shift of the heparin fragment, while 176SSS178 and 187SCS189 induced minor shifts of the heparin fragment. These observations support the previous studies indicating the importance of lysine residues in binding GAGs. However, the linker BXBB motif and the helical lysine residues contribute more to DBPA B31’s
GAG affinity than the C-terminal basic clusters. Specifically, the $^{176}\text{SSS}^{178}$ mutant has a larger effect on the binding than the $^{187}\text{SCS}^{189}$ mutant. Similar trends were seen with heparin dp6 (Figure 2.5); although, its interactions with the C-terminal mutants of $^{176}\text{SSS}^{178}$ and $^{187}\text{SCS}^{189}$ are not readily visualized as in heparin dp10, indicating the binding affinity of DBPA for heparin dp6 may be weaker.

**Figure 2.5.** Gel mobility shift assay of heparin dp6 in the presence of increasing concentrations of (A) B31 WT, $^{64}\text{SDSS}^{67}$, $^{176}\text{SSS}^{178}$, and $^{187}\text{SCS}^{189}$ and of (B) B31 WT, $^{64}\text{SDSS}^{67}$, and K82,170S. The same assay was also conducted with DS dp6 (Figure 2.6). However, its weak affinity for DBPA demanded the use of conditions more conducive to protein-GAG interactions. In particular, the assay was conducted at pH 5 and at a GAG-to-protein ratio of 3. Under
these conditions, some DS binding can be seen for the wild type and the $^{187}\text{SCS}^{189}$ mutant. However, the other three mutants showed no sign of binding DS dp6. This is consistent with what has been observed with heparin fragments, indicating DBPA’s interactions with DS and heparin are governed by similar factors.

![Gel mobility shift assay of DS dp6 in the absence and in the presence of B31 WT, 64SDSS, 67K82,170S, 176SSS, and 187SCS.]

**Figure 2.6.** Gel mobility shift assay of DS dp6 in the absence and in the presence of B31 WT, 64SDSS, 67K82,170S, 176SSS, and 187SCS.

### 2.3.2 Thermodynamic Contributions of the GAG-Binding Motifs

Titrations were conducted with heparin dp6 on the WT and mutant forms of B31 to quantitatively measure the thermodynamic contribution of each motif to GAG binding. Because GAG-protein interactions are highly dependent on the size of the GAG polymer, B31 DBPA’s affinity for heparin dp6 is weak at physiological pH ($K_D \sim 4$ mM). Such weak affinity made detection of affinity changes difficult. As a result, the titrations were repeated at pH 5.0, a pH that produced more favorable electrostatic surface potentials for binding, thus increasing the affinity of DBPA for heparin. Furthermore, the pH change did not change the pattern of the peaks or the peak migration direction significantly, indicating DBPA-heparin interactions were not greatly perturbed. With the lower pH,
binding of heparin dp6 to WT B31 went from a $K_D$ of 4 mM to a $K_D$ of 0.5 mM (Figure 2.7).

**Figure 2.7.** $^1$H–$^{15}$N HSQC overlays of DBPA B31 WT in the presence of increasing concentrations of heparin dp6. Signals experiencing a large migration (E186 and N191) are indicated with the residue number and direction of migration. The red contour represents the initial HSQC spectrum of DBPA in the absence of heparin dp6. Each subsequently colored contour represents the HSQC spectrum of DBPA at different concentrations of heparin dp6. The concentrations of heparin dp6 are 0.3, 0.6, 0.9, 1.2, 1.5, and 2.4 mM. The concentration of DPBA is 0.15 mM.

The shifts of two C-terminal residues (E186 and N191) showing the largest migration distances were used to determine the $K_D$ of the interaction for each DBPA variant. The only exception was mutant K82,170S, for which A54 was used in place of E186 because of the poor signal-to-noise ratio of the E186 peak. Table 2.1 lists the $K_D$ values derived from N191 and E186 for each B31 variant. It is noteworthy that $K_D$ values obtained using E186 chemical shift migrations are consistently lower than those from N191. This most likely reflects the flexibility of the C-terminus as a binding site and the fact that acidic
amino acids are more likely to repel the negatively charged GAG ligands, thus providing a higher $K_D$. However, these E186-derived $K_D$ values reflect the same trend of decreasing affinities among the mutants examined that those of N191 do.

Table 2.1. $K_D$ of DBPA-heparin dp6 interaction from calculation using chemical shift changes from residues E186 and N191 of DBPA B31 variants as well as from ITC.

<table>
<thead>
<tr>
<th>B31 Mutant</th>
<th>$K_D$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N191</td>
</tr>
<tr>
<td>WT</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>$^{64}$SDSS$^{67}$</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>K82,170S</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>$^{176}$SS$^{178}$</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>$^{187}$SC$^{189}$</td>
<td>0.85 ± 0.06</td>
</tr>
</tbody>
</table>

Compared to the binding $K_D$ of 0.5 mM for the wild type, the linker mutant $^{64}$SDSS$^{67}$ exhibited a much higher $K_D$. An accurate value of the $K_D$ for the $^{64}$SDSS$^{67}$ mutant could not be obtained under the current experimental conditions but is estimated to be >20 mM. The $K_D$ values for helical mutant K82,170S and C-terminal mutants $^{176}$SS$^{178}$ and $^{187}$SC$^{189}$ were approximately 4, 2.5, and 0.85 mM, respectively. The binding curves for all samples are included in Figure 2.8.
Figure 2.8. \( K_D \) curves of two residues for B31 WT and mutants. These residues (A54, E186, and N191) experienced the greatest linear peak migration when titrated with heparin dp6, and these peaks were analyzed to give the \( K_D \) for each B31 variant. Residue A54 was used in place of E186 for the K82,170S mutant due to poor signal-to-noise from E186 of the mutant.
On the basis of these dissociation constants, the ΔG contributions of the BXBB motif in the linker were calculated to be more than 2.7 kcal/mol while the K82,170S motif and the C-terminal 176SS178 motif contribute only 1.2 and 1 kcal/mol, respectively. The 187SCS189 motif contributes very little thermodynamically to GAG binding. A summary of the results is presented in Table 2.1.

To confirm the NMR observations and shed further light on the thermodynamic driving force of DBPA-GAG interactions, we also performed ITC analysis of their interactions between heparin dp6 and WT DBPA as well as two weaker binding mutants, 64SDSS67 and K82,170S. The ITC results agreed well with the NMR findings. In particular, ITC found the K_D for the complex of DBPA WT B31 with heparin dp6 to be ~0.8 mM. Estimations from ITC about the K_D of the interactions of heparin dp6 with the 64SDSS67 mutant and the K82,170S mutant are less precise because of limitations on the concentrations of both the heparin dp6 stock solution, which produced a high dilution heat at high concentrations, and the mutants, but both titrations showed clear signs of significant increases in their K_D values. The K_D of interactions of heparin dp6 with the 64SDSS67 mutant is estimated to be ~5 mM. The K_D for the K82,170S mutant is estimated to be ~10 mM (Table 2.1 and Figure 2.9). These results agree with the trend obtained using GMSA and NMR. Furthermore, similar to those of other carbohydrate-binding proteins [51], DBPA’s interaction with heparin dp6 is mostly driven by favorable enthalpic changes, which is estimated to be approximately -4.8 kcal/mol. Entropic changes were small but unfavorable, contributing approximately 0.5 kcal/mol to the free energy change. Figure 2.9 shows the ITC titration curves of the WT, as well as 64SDSS67 and K82,170S mutants, with heparin dp6.
2.3.3 Specific DBPA-GAG Interactions Using Paramagnetically-Labeled GAG Ligands

In our previous characterization of B31 DBPA structure, indirect indications of the flexible linker’s role in contacting GAGs were already evident. In particular, NMR chemical shifts of atoms in linker residues underwent significantly larger changes than residues in other parts of the protein, and amide protons of residues G69, S70, and G71 saw dramatic decreases in solvent exchange rates in the presence of heparin dp6 [21]. To obtain direct evidence that the linker is close to the bound GAG, we probed B31 DBPA with a novel paramagnetic heparin dp6 ligand. The paramagnetic functional group used in this study is TEMPO, a stable nitroxide radical that has commonly been used as a structural probe in solution NMR. The paramagnetic effect of TEMPO stems from the unpaired electron that generates an inhomogeneous magnetic field in its vicinity, causing NMR signals of nearby atoms to experience larger longitudinal and transverse relaxation rates, leading to signal broadening or PRE. Because PRE is distance-dependent, the residues closest to the radical will experience the greatest increases in relaxation rates,
which can be quantitatively measured [45]. TEMPO-tagged glycans have been used previously to probe protein-glycan interactions [52, 53]. However, there is no report of location-specific TEMPO labeling of GAG fragments for the purpose of studying protein-GAG interactions.

For this study, we constructed a novel TEMPO-labeled heparin dp6 derivative by attaching 4-amino-TEMPO specifically to the reducing end of the heparin dp6 fragment using reductive amination (Figure 2.3). Although reductive amination has been used to attach fluorescent tags to heparin, this is the first report of stable radical functionalization of heparin using reductive amination. To accurately quantify the size of the PRE on each residue, the amide proton transverse relaxation rates were measured in the presence of six molar equivalents of TEMPO-labeled heparin dp6 before and after reducing the TEMPO radical with ascorbic acid. The difference in the two relaxation rates is due entirely to the PRE from the nearby radical. This allowed the location of the reducing end of heparin to be accurately determined. Figure 2.10A shows the residue-specific PRE of each backbone amide proton.
Figure 2.10. (A) Comparison of residue-specific PRE effect on the backbone amide proton from TEMPO-labeled heparin dp6 before and after reduction of the TEMPO radical. The comparison indicated that two residues (N59 and F60) experienced a greater PRE effect when probed with the TEMPO radical. (B) Ribbon depiction of DBPA B31 WT with the residues experiencing the greatest PRE effect colored yellow.

The residues that showed the most perturbation after the radical was reduced were N59 and F60, both of which showed a rate decrease of >20 s\(^{-1}\). Figure 2.11 shows the HSQC peaks of the two residues in the presence of the oxidized and reduced radical as well as the normalized intensity decay curve from which the relaxation rates were calculated. These residues are positioned near the BXBB epitope in the linker. Because the radical was specifically attached to the reducing end of the heparin fragment, the PRE data imply that the reducing end of the fragment is present near the BXBB epitope as well. These data provide direct confirmation that the BXBB motif is part of the GAG-binding site of DBPA.
It is also noteworthy that other basic amino acid clusters in the protein showed minimal perturbation by the TEMPO-labeled heparin. However, this may be because these residues interact only with regions of heparin distant from the reducing end. To confirm the PRE effect is not the result of non-specific TEMPO-DBPA interactions, the protein was also titrated with 4-amino-TEMPO at similar protein-to-ligand ratios, and no significant changes in the transverse relaxation rates of the protein were observed.

2.3.4 Backbone Dynamics of B31 DBPA

Because both the linker and the C-terminus of DBPA are quite flexible, the possibility of GAG-induced changes in backbone dynamics of the protein exist. To
quantify these changes, the popular Model-Free approach was used to estimate the extent of picosecond to nanosecond time scale internal motions in the protein [54, 55]. This technique allowed the magnitude of internal motions of the atoms to be represented by the simple order parameter $S^2$. Furthermore, its value can be estimated using measurable NMR observables such as longitudinal and transverse relaxations rates and steady state heteronuclear NOE [49]. These observables were measured on the backbone amide nitrogen atom using established NMR experiments, and the experimental data were fit using relax [48] to obtain residue-specific order parameters. The fitting assumes the protein undergoes isotropic global rotational motion, which is usually true for well-folded globular proteins. To perform the fitting, the global rotational correlation time ($\tau_m$) of the protein was estimated using transverse and longitudinal relaxation rates of residues located in the structured parts of the protein. In the absence of heparin dp6, $\tau_m$ was estimated to be ~12.5 ns. After 24 equivalents of heparin dp6 had been added, $\tau_m$ increased slightly to 14.2 ns. Figure 2.12 shows the order parameters of backbone amide nitrogen atoms of WT B31 in the presence and absence of 24 equivalents of heparin dp6. An order parameter value of 0 indicates a complete lack of internal order, while a value of 1 indicates the atom is perfectly rigid relative to other atoms and experiences no internal motion. The values of order parameters for the structured parts of the protein are around 0.85 (Figure 2.12).
Figure 2.12. Order parameter of backbone amide nitrogen atoms for DBPA B31 WT in the absence (——) and presence (---) of 24 molar equivalents of heparin dp6. Details of order parameter changes for residues in the first half of the flexible linker are shown in the right panel.

No large changes in order parameters were observed for the heparin-containing sample. However, some reductions in order parameters were observed for residues in the first half of the linker. In particular, residue F60, which is one of the two residues greatly perturbed by the paramagnetic ligand, showed an increase of ~0.15 in its $S^2$ value after heparin dp6 had been added. These measurements indicate the presence of heparin dp6 did not change the magnitudes of DBPA’s fast time scale motions significantly. The lack of large-scale changes in protein dynamics is consistent with ITC measurements, which showed entropic contributions to binding are small. To ensure the accuracy of the fitting, contributions to observed relaxation rates from microsecond to millisecond time scale conformational exchange ($R_{\text{ex}}$) were also estimated in the model. However, no significant contributions from motions on these time scales were seen.
2.4 Discussion

Since the discovery of DBPA, its role in the development of Lyme disease has been extensively studied [23, 24, 27, 56]. It has been shown to be important in the establishment of infection in early stages of the process and may act by both anchoring bacteria to the extracellular matrix and modulating the immune system response to the bacterium [24]. One aspect of DBPA’s activity that has not been fully explored is the relationship between variations in its sequences and its activity as an adhesin. In particular, a previous study revealed that DBPAs from strains B31 and 297 of *B. burgdorferi* possessed much higher GAG affinities than strains N40 and B356 despite a high level of sequence homology among them [19].

**Figure 2.13.** Sequence alignment of DBPA variants found in B31, 297, N40, PB and VS461 strains for *Borrelia*. Strain B356 is not shown because it has 99% sequence identity with strain N40.

Our results offer a possible explanation for the following observations: both B31 and 297 DBPAs contain the BXBB motif in the linker; whereas in N40 and B356, the motif is
substituted with the sequence, TDSE (Figure 2.13), making the net charge for the cluster -2 rather than +2. This effectively prevented strong interactions between the linker and GAGs. Besides the changes in the linker, N40 and B356 strains of DBPA are also devoid of residues equivalent to K124 and K128 of B31. However, these residues are not located in the binding pocket and have not been perturbed significantly in either chemical shift mapping or the PRE experiments and, thus, may not play a significant role in GAG binding. The fact that DBPA from strain 297 has a high affinity for GAGs despite not having a residue equivalent to K128 also partially confirms the hypothesis. The differences in GAG-binding affinity between B31 and N40 DBPAs cannot be attributed to a lack of basic amino acids in N40 DBPA either: N40 has a number of basic amino acids in its version of DBPA comparable to the number in B31’s version (27 basic amino acids in N40 vs. 29 in B31) as well as a basic-to-acidic residue ratio (1.08) similar to that of B31 (1.07). Therefore, the lack of the BXBB motif could be a major factor in N40 DBPA’s lower GAG affinity. In fact, this variation in DBPA sequence could be a significant contributing factor to the observed lower binding efficiency to host cells by the N40 strains of *Borrelia* [57]. An analysis of all DBPA sequences from *B. burgdorferi* available in the UniProt database showed that of 20 available sequences, seven possessing the BXBB motif, indicating the GAG-binding enhancing epitope is not exclusive to strains B31 and 297.

In this work, the role played by the BXBB motif in GAG binding has been experimentally verified using paramagnetically-tagged GAG ligands. In addition to providing confirmation of interactions between the BXBB motif and GAGs, results from the titration of DBPA by the paramagnetic ligand are surprising in that they show
DBPA’s interactions with GAGs may be highly orientation-specific. The fact that only residues in the linkers experienced significant PRE indicates the reducing end of the ligand is close to the linker. However, the C-terminus, which is located at the opposite side of the binding pocket, may not be. An alternative explanation is that the affinity of the C-terminus for GAGs is weaker and, therefore, may not be affected by PRE to the same extent as the linker residues when the reducing end is close to it. Consequently, the binding mode with the fragment in the opposite orientation may not be detected by PRE. It is also unclear whether the specificity is a consequence of the opening of the reducing end monosaccharide during reductive amination. The increased flexibility due to the linearization of the sugar may have artificially increased the affinity of the pockets for the reducing end. However, if the observation is not the result of an artifact, it shows DBPA’s interactions with GAGs are highly specific and may be the result of close geometric matching between sulfate groups on GAGs and basic amino acids in DBPA.

Characterization of DBPA’s backbone dynamics showed conformational entropy does not appear to be a significant factor in determining the binding affinity. This agrees with the small entropic change measured in ITC. The fact that one of the most flexible segments of the protein also contains a crucial GAG-binding epitope is demonstrative of the dynamic nature of DBPA-GAG interactions and lends a plausible explanation for the weak interaction of DBPA with GAGs. However, despite the millimolar dissociation constants for these interactions, the interaction is by no means irrelevant. The dissociation constants measured in this study pertain to only low-molecular weight heparin fragments. However, it is well-known that the affinity of proteins for GAGs is highly dependent on the size of the GAGs. Longer GAG polymers in vivo will have a much higher affinity for
DBPA than the hexasaccharide used in this study because of the effects of avidity. This does not imply the use of short GAG fragments as the ligand is not relevant. The size of the GAG-binding pocket on DBPA can accommodate only one hexasaccharide at a time; therefore, hexasaccharides are the right size to achieve sufficient affinity without the risk of promoting protein oligomerization. Size-defined GAG fragments also allowed for a more objective evaluation of DBPA’s preferences for different GAG types. In fact, such weak interactions are by no means extraordinary in protein-carbohydrate interactions [4]. Specifically, the well-studied interactions between the influenza A viral hemagglutinin monomer and its sialylated N-glycan receptor possess dissociation constants in the millimolar range [58], as does the interaction between high-mannose N-glycan and the immune receptor DC-SIGN [59]. These weak interactions are still relevant because most protein-carbohydrate interactions rely on multivalency and avidity effects to achieve sufficient binding affinity. Both factors should play a role in DBPA-mediated interactions with GAGs.

Although the GAG-binding site composed of the linker residues and the C-terminus is the highest-affinity site on DBPA, titrations with conventional GAG ligands have also revealed that the N-terminus may be a weaker secondary binding site. These interactions are manifested in the significant chemical shift changes in atoms from N-terminal residues T28 and T101 during heparin dp6 titrations (Figure 2.7). However, $K_D$ fitting showed that the dissociation constant of the binding is much weaker than that of residues located in the high-affinity site (3 mM vs. 0.5 mM; Figure 2.14), and the mutations at the main site left the $K_D$ at the N-terminal site unchanged, indicating the N-terminal site is independent of the main GAG-binding site.
Figure 2.14. $K_D$ curves of N-terminal residues T28 and T101 of WT B31.

The catalyst of these interactions is most likely a cluster of basic amino acids at the N-terminus, including R34, K102, and K104. The helical conformation of the segment allowed them to form a basic strip at the N-terminus that offers optimal geometry for the interactions with GAGs. HSQCs of Arg sidechain $H_{\varepsilon} - N_{\varepsilon}$ showed the sidechain of R34 experienced significant changes in chemical shift and signal intensity in the presence of heparin dp6 fragments (data not shown). This provides further proof that the N-terminus is involved in GAG binding. Although it has a weaker affinity than the main GAG-binding site, the secondary binding site may still offer significant contributions to DBPA’s interactions with native GAG polymers in vivo.
CHAPTER 3

Structural Mechanisms Underlying Sequence-Dependent Variations in GAG-Affinities of Decorin Binding Protein A, a *Borrelia burgdorferi* adhesin


3.1 Introduction

Although the GAG-binding epitopes of B31 and N40 versions of DBPA have been investigated [50, 60], very little is known about the GAG-binding epitopes of PBr DBPA and the mechanisms by which it achieves its higher GAG affinities. Knowing the mechanisms leading to the GAG affinity differences among DBPA variants are not only important to understanding the role of this protein in promoting bacterial adhesion, but it may also help in designing DBPA-based tools for combating *Borrelia* bacteria. In particular, because of DBPA’s strong tendency to induce human immune response, it has been considered as a component in vaccines and serological tests for Lyme disease [61]. However, the large sequence variation in DBPA may require the inclusion of several versions of DBPA to elicit comprehensive immunity against all strains since sequence variations in DBPA may prevent antibodies against DBPA of one strain from detecting other versions. Understanding of the structural differences and similarities among the different strains will aid in the intelligent selection of representative DBPA variants that can present greatest coverage in these tests. The family of DBPA proteins also provides an excellent opportunity to study diversity in GAG-binding motifs. Understanding of the structure-activity relationships of GAG-binding proteins is still rudimentary. DBPAs share a similar tertiary fold; therefore, GAG affinity differences should come mainly
from differences in arrangements of GAG-binding residues on this common frame. This allows the effects of spatial arrangement of basic amino acids on the protein’s affinity and specificity for GAGs to be investigated.

Previously, structure-activity relationships of DBPAs from the closely related strains of B31 and 297 have been investigated by us and other groups [21, 29, 60]. To further understand the correlation of DBPA sequence variation with differences in GAG affinities, we have determined the solution structures for N40 and PBr strains of DBPA and analyzed the interactions of these proteins with GAG ligands. The structures of both proteins retain the five helical bundle fold of B31 DBPA, with the N40 DBPA structure being almost identical with that of B31 DBPA. However, the structure of PBr DBPA contains significant differences compared with B31 DBPA. In particular, the flexible linker between helices one and two adopts a helical conformation in PBr DBPA whereas the same segments in B31 and N40 are mostly unstructured. In addition, the C-terminal tail of PBr DBPA has a different orientation than those found in B31 and N40 DBPAs. The functional consequences of these changes are that, unlike B31 and N40 DBPAs, which contain only a single GAG-binding epitope, PBr DBPA contains at least two independent binding epitopes. Whereas the location of one epitope is similar to the major GAG-binding epitopes found in B31 and N40 versions of the protein, the other epitope in PBr DBPA is located on the opposite side of the helical bundle, outside the canonical binding pocket for DBPA. Quantitative analysis of binding dissociation constants using NMR and other techniques showed that the new epitope has as high an affinity for GAGs as the epitope in the traditional binding pocket and the two epitopes are completely independent of one another. Our results show that the mechanisms of GAG binding vary
greatly among different DBPA variants and detailed structural study will be required to fully understand the GAG-binding mechanisms of each.

3.2 Experimental Procedures

3.2.1 Expression and Purification of B31, N40 and PBr DBPAs

The ORFs for the WT mature DBPA strains B31 (residues 24-191), N40 (residues 29-194) and PBr (residues 22-185) were synthesized by GenScript Inc. Each ORF was cloned into the pHUE vector which results in a fusion protein of His-tagged ubiquitin at the N-terminus of each DBPA [42]. PBr mutants were created using site-directed mutagenesis from the WT sequence. The forward primers for Site 1 and Site 2 are the following: K79S, 5'-GCCGGAATTTATCTGAAAGCCAGCATTTAAAGCAATCCAAGTGGC-3'; K130S, 5'-CTGGAAGAAATTGCGATCCAGAGCATGACCGGTACGGTG-3'; K160S, 5'-GCCCAGGCAATGGAAGACAGCCTGAACATGTCAAC-3'; K77,81S, 5'-CCGGAATTTATCCTGAGCGCCAAGATTAGCGCAATCCAAGTGCTG-3'; K173,176S, 5'-CAACATGATGCCTGAGCAATCTGAGCGAAAAAGCTAAGACCGCG-3'.

Incorporation of the correct mutations was confirmed through sequencing.

Each plasmid was transformed into *E. coli* BL21(DE3) and then the bacteria were grown at 37°C to an OD_600_ of 0.5. Unlabeled protein was grown in normal Luria-Bertani (LB) broth whereas isotopically labeled protein was grown in M9 medium supplemented with ^15^NH_4^Cl or [^13^C]-glucose. The bacteria were induced with 0.5 mM IPTG and incubated overnight at 30°C. After the cells had been harvested, the resuspended pellet was incubated with 1 mg/mL lysozyme and sonicated to lyse the cells. The fusion protein
was extracted from the cleared supernatant via Ni-affinity chromatography with a 5 mL HisTrap column (GE Life Sciences) and was eluted using an imidazole gradient of 25-300 mM at a flow rate of 3 mL/min. The fusion protein was collected and exchanged into 25 mM Tris (pH 8.0), 100 mM NaCl and was digested with USP2 and 1 mM DTT overnight at room temperature. The cleaved DBPA was separated using a 5 mL HisTrap column and was eluted in the flow-through which was then collected, concentrated and exchanged into 20 mM acetate pH 5.0, 150 mM NaCl.

3.2.2 Acquisition and Analysis of NMR Structural Data for N40 and PBr DBPAs

NMR data for N40 and PBr structure determination were collected on Varian Inova 800 MHz, Bruker Ultra-Shield 600 and 850 MHz spectrometers. Many of the pulse sequences were included in the pulse sequence packages BioPack and TopSpin. HNCACB, CBCACONH, HNCOCA and HNCO experiments were collected for backbone atom assignment. For sidechain atom assignments, CCONH, HCCONH, HCCH—TOCSY, $^{15}\text{N}$-edited TOCSY and $^{13}\text{C}$- and $^{15}\text{N}$-edited NOESY—HSQC spectra were collected. Both HN and NC residual dipolar couplings (RDCs) were collected using J-modulated pulse sequence on both proteins aligned in 6% polyacrylamide gel [62]. Data processing was conducted with NMRPipe [46] and analysis was done with NMRView [47].

Relaxation and PRE experiments were collected on Bruker Ultra-Shield 600 MHz and Varian Inova 800 MHz spectrometers. T1, T2, and steady-state heteronuclear NOE experiments were collected for both WT N40 and PBr with and without 24 molar equivalents of heparin dp6 (degree of polymerization 6, i.e. hexasaccharide). NMRPipe was used to process and NMRView was used to analyze the data. The order parameters
(S^2) were extracted for each protein using relax Model-Free software [48]. PRE of TEMPO-labeled heparin dp6 was measured from ^1^H T_2 of backbone amide protons [45]. For the N40 PRE experiment, 6 molar equivalents of TEMPO-labeled heparin dp6 was added to 400 µL of 150 µM protein whereas two molar equivalents were added to 400 µL of 300 µM PBr. Contribution of the TEMPO radical was seen in the difference between ^1^H T_2 before and after reduction of the radical with ten molar equivalents of ascorbate.

3.2.3 Structure Determination for N40 and PBr DBPAs

Both ^13^C- and ^15^N-isotopically edited NOESY—HSQC data were analyzed and assigned, primarily to identify long-range contacts. Backbone dihedral angles of the α-helical residues were calculated using TALOS [63]. The NOESY—HSQC peak lists, as well as the dihedral angles, were used in CYANA to determine each structure [64]. The resulting structure with minimal long-range violations, along with RDCs, was refined in XPLOR-NIH [65]. Surface electrostatic potential of N40 and PBr DBPAs at an ionic strength of 150 mM were calculated with the program APBS [66].

3.2.4 Production and Modification of Heparin and Heparin Fragments

Heparin (Sigma-Aldrich) was dialyzed and lyophilized to remove excess salt prior to being digested with heparinase I. Digestion was allowed to continue until depolymerization was approximately 30% complete to give fragments of varying size. These fragments were separated via size exclusion chromatography on a 2.5 cm × 175 cm column (Bio-Rad Biogel P10) with a flow rate of 0.2 mL/min. Each fragment was collected, dialyzed and lyophilized prior to HPLC verification of fragment size. The fragments were quantified gravimetrically or with a carbazole assay [67]. The reducing
end of heparin dp6 was modified with 4-amino-TEMPO through reductive amination [60]. This TEMPO-labeled GAG fragment was used for the PRE study.

3.2.5 Titrations of DBPA Variants with Heparin dp6

NMR-monitored titration was used to estimate the $K_D$ values for the interaction between heparin dp6 and DBPA variants analyzed in the present study. For each PBr titration, fourteen molar equivalents of heparin dp6 were added to 400 µL of 150 µM protein in two molar equivalent aliquots; whereas, the titration of N40 involved the addition of twenty-five molar equivalents of heparin dp6 to 400 µL of 150 µM protein in 5 molar equivalent aliquots. This was conducted in 150 mM NaCl, 20 mM acetate buffer at pH 5.0 for WT N40, WT PBr, as well as PBr Site 1, Site 2, Site 2a and Site 2b. The chemical shift changes noted in each $^1$H—$^{15}$N HSQC were normalized into one chemical shift value using Equation 1.1 [35]. The $K_D$ binding curves were fitted in xcrvfit (http://www.bionmr.ualberta.ca/bds/software/xcrvfit/) to plot the normalized chemical shift against the heparin dp6-to-DBPA ratio. $^1$H—$^{15}$N HSQC spectra were collected on a Bruker Ultra-Shield 600 MHz spectrometer.

3.2.6 Gel Mobility Shift Assays for WT B31, N40, PBr and PBr Mutants

Heparin dp8 fragments were fluorescently-labeled with 0.1 M 2-AMAC according to the method of Lyon et al. [32]. The WT DBPA assay was performed by mixing 1 µg of the fluorescently-labeled heparin dp6 with one-half, one or two molar equivalents of either B31, N40, or PBr DBPA in 50 mM sodium phosphate (pH 6.5), 150 mM NaCl buffer in a total volume of 12.5 µL. The PBr mutant assay was performed by mixing 1 µg of the fluorescently-labeled heparin dp8 with one or two molar equivalents of DBPA in 50 mM sodium phosphate (pH 6.5), 150 mM NaCl buffer in a total volume
of 12.5 µL. The control for both assays is protein-free fluorescent heparin dp6 or dp8 fragments. The reaction mixtures were incubated at room temperature for thirty minutes then were run in a 1% agarose gel at 120 V for fifteen to twenty-five minutes. A UV panel was used to visualize the shifts [44].

3.2.7 ELISA Analysis of Heparin Binding to WT PBr and PBr Mutants

Intact heparin was biotinylated and quantified with the Biotinylation Quantitation kit (Pierce) according to manufacturer’s instructions. Neutravidin-coated microplates (G-Biosciences) were coated with 100 µL of PBS containing 20 µg/mL of biotinylated heparin and were incubated with 2 µg of His-tagged WT PBr and PBr mutants in 100 µL of PBS. The results were quantified using 1:2000 anti-His HRP (Qiagen) and developed with tetramethylbenzidine (TMB) substrate solution. An additional 100 µL of 0.1 M HCl was added to each well to stop the reaction which induced a color change thus allowing the plate to be read at 450 nm.

3.2.8 Competitive Heparin-Binding ELISA for PBr DBPA Variants and Biotinylated N40 DBPA

N40 DBPA was biotinylated by reacting carboxy sidechains in N40 DBPA with amino-PEG2-biotin (Thermo Scientific). Specifically, 1 mL of 26 µM N40 DBPA was treated with 2.6 mM EDC and 5.2 mM amino-PEG2-biotin for thirty minutes at room temperature. Excess biotin and EDC were then removed by desalting the mixture with a HiTrap Desalting column (GE Healthcare). The competition ELISA was carried out by first incubating commercially available heparin-coated microwell plates (Bioworld) with 100 µL per well of PBS containing 4 µg, of biotinylated N40 DBPA and either 0, 2, 4, or 6 µg of PBr DBPA variants for one hour. The amount of bound N40 DBPA was then
determined by incubating the wells with streptavidin-HRP (Cell Signaling Inc.) for one hour and followed by treatment with TMB reagents for twenty minutes before the absorbance at 450 nm was measured.

3.3 Results

3.3.1 Structural Differences Among DBPAs of Strains B31, N40 and PBr

Solution structure of B31 DBPA comprises a five-helix bundle with unstructured segments between the first two helices and at the C-terminus [21]. The crystal structure of DBPA from strain 297 adopts an identical fold [29]. Building on these investigations, we have determined the solution structures of DBPA from strain N40 of *B. burgdorferi* and strain PBr of *B. garinii*, two variants that have significantly different GAG affinities than B31 DBPA [19, 26]. Structural statistics for these two structures are shown in Table 3.1.
Table 3.1. Structural statistics for the ensemble of N40 and PBr structures.

<table>
<thead>
<tr>
<th></th>
<th>N40</th>
<th>PBr</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of NOE-based distance constraints</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1732</td>
<td>1440</td>
</tr>
<tr>
<td>Intraresidue (i = j)</td>
<td>351</td>
<td>398</td>
</tr>
<tr>
<td>Sequential (</td>
<td>i – j</td>
<td>= 1)</td>
</tr>
<tr>
<td>Medium-range (1 &lt;</td>
<td>i – j</td>
<td>&lt; 5)</td>
</tr>
<tr>
<td>Long-range</td>
<td>494</td>
<td>230</td>
</tr>
<tr>
<td>NOE constraints per restrained residue(^a)</td>
<td>10.7</td>
<td>8.8</td>
</tr>
<tr>
<td>No. of dihedral angle constraints</td>
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<td>280</td>
</tr>
<tr>
<td>Total no. of structures computed</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>No. of structures used</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Residual constraint violations(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of distance violations per structure</td>
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<td></td>
</tr>
<tr>
<td>0.1-0.5 Å</td>
<td>32.2</td>
<td>41.3</td>
</tr>
<tr>
<td>&gt;0.5 Å</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No. of dihedral angle violations per structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-10(^\circ)</td>
<td>5.6</td>
<td>9.0</td>
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<tr>
<td>&gt;10(^\circ)</td>
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<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>All backbone atoms</td>
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</tr>
<tr>
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<td>2.5 Å</td>
</tr>
<tr>
<td>Ordered(^c)</td>
<td>0.7 Å</td>
<td>0.6 Å</td>
</tr>
<tr>
<td>All heavy atoms</td>
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<td></td>
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<tr>
<td>All</td>
<td>1.7 Å</td>
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<td>Ordered(^c)</td>
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<td>1.1 Å</td>
</tr>
<tr>
<td>Ramachandran plot summary from Procheck(^d) (%)</td>
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<tr>
<td>Most favored regions</td>
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<td>Additionally allowed regions</td>
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<tr>
<td>Disallowed regions</td>
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<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\) There are 162 residues (for N40) and 163 residues (for PBr) with conformationally restricting constraints. \(^b\) Calculated for all constraints for the given residues, using sum over r\(^b\). \(^c\) Residues with sum of phi and psi order parameters > 1.8. Ordered residue ranges for N40 are the following: 35-59, 79-104, 112-133, 136-148 and 152-183. Ordered residue ranges for PBr are the following: 34-52, 73-100, 106-126, 130-141 and 147 to 179. \(^d\) Residues selected on basis of dihedral angle order parameter, with S(\(\phi\))+S(\(\psi\)) ≥ 1.8. Selected residue ranges for N40 and PBr are the same as the ordered residue ranges.

It is evident from these structures that N40 and PBr DBPAs have similar helical topologies and hydrophobic packings as B31 DBPA (Figure 3.1).
Figure 3.1. Ensemble of the 10 lowest-energy structures of (A) N40 DBPA and (B) PBr DBPA in solution. Helix 1 (residues 33-60 for N40, residues 30-50 for PBr) is colored green. Helix 2 (residues 79-105 for N40, residues 72-99 for PBr) is colored blue. Helix 3 (residues 112-131 for N40, residues 106-126 for PBr) is colored red. Helix 4 (residues 137-145 for N40, residues 131-141 for PBr) is colored cyan. Helix 5 (residues 157-183 for N40, residues 147-174 for PBr) is colored purple. The structured linker (residues 56-67) of PBr is colored yellow, and the disulfide bond anchoring the C-terminus to helix 5 in N40 is colored orange. The schematic topology of N40 is shown at the bottom left and PBr, the bottom right.

Sequence alignment of all three DBPAs indicates that, despite the low sequence identity between PBr and B31 DBPAs, all three DBPAs share remarkably similar secondary structural element arrangements (Figure 3.2).
**Figure 3.2.** Sequence alignment of B31, N40, and PBr DBPAs. The helical regions of each protein are highlighted in black. The structured linker found in PBr DBPA is notated in grey.

Analysis of hydrophobic contacts within each structure showed that both N40 and PBr DBPAs are stabilized by the presence of a hydrophobic core formed with hydrophobic amino acids from helices two, three and five. The same hydrophobic core is also present in B31 DBPA.

Figure 3.3 shows the superimposition of B31, N40 and PBr DBPA structures. Both N40 and PBr DBPA structures superimposed with the B31 DBPA structure very well. RMSD between helical residues of B31 DBPA and N40 DBPA is 1.9 Å and RMSD between helical residues of B31 DBPA and PBr DBPA is 2.3 Å. Despite the similarities in the helical regions, structure of PBr DBPA differs significantly from B31 and N40 DBPAs in two respects. Specifically, the linker between helices one and two of PBr DBPA (residues 56-67) adopts a helical conformation whereas the same region in B31 and N40 DBPA is mostly unstructured (Figure 3.3).
Figure 3.3. Superimposition of B31 (cyan), N40 (green) and PBr (tan) DBPA structures. The linker in PBr DBPA is more retracted than the linkers in B31 and N40 DBPAs. The C-terminus in PBr DBPA is also not tethered to helix 5 by a disulfide bond as it is in B31 and N40 DBPAs.

The helical nature of PBr DBPA’s linker was confirmed by the values of backbone dihedral angles of the linker residues predicted using TALOS+ [68] and by the presence of NOEs between sequential amide protons, which are reliable indicators of helical conformation. The structural consequence of such a conformation change is that the PBr DBPA linker is more compact than those in B31 and N40 DBPAs. In addition, PBr does not contain the disulfide bond connecting the C-terminus and helix 5, which is present in both B31 and N40 DBPAs. This allows the C-terminus of PBr to be less restricted and to point away from the canonical GAG-binding pocket (Figure 3.3). It should also be noted
that both N40 and PBr are missing the basic amino acid cluster (the BXBB motif) in the flexible linker, which is known to be important for GAG binding in B31 DBPA [60].

Because GAG-protein interactions are mediated mostly by electrostatic interactions between sulfate or carboxyl groups on the polysaccharide and basic amino acids on the protein, a surface electrostatic potential map of the protein is often a good way to identify possible GAG-binding epitopes. Electrostatic potential mappings of N40 and PBr DBPAs confirmed the presence of a basic pocket formed by helices 1, 2 and 5 (Figures 3.4 and 3.5) of both proteins.

**Figure 3.4.** Basic pocket of N40 DBPA. (A) Electrostatic potential surface map of N40 DBPA. Red indicates acidic regions and blue, basic regions. (B) Ribbon diagram of N40 DBPA showing the protein in the same orientation as Figure 1A.

This basic patch, which contains three lysine residues (K82, K163 and K170 in B31 DBPA; K85, K166 and K173 in N40 DBPA) known to be crucial to GAG binding, has also been identified as the primary site for DBPA-GAG interactions in both B31 and 297 DBPAs [29, 50, 60]. It is notable that the linker in N40 and B31 DBPAs almost entirely
obscribes this basic pocket. However, the helical nature of the linker in PBr results in a more exposed basic patch than in B31 and N40 DBPAs (Figure 3.5).

Figure 3.5. Electrostatic potential surface maps of PBr DBPA in the same orientation as Figure 1B (A) and rotated 180° about the vertical axis (C). Red indicates acidic regions and blue, basic regions. Ribbon diagrams of PBr DBPA showing the protein in the same orientation as Figure 1B (C) and rotated 180° about the vertical axis (D).
Besides the three conserved lysines (K79, K160, and K167) in the canonical binding site (Site 1), the electrostatic potential map of PBr also indicates the possible existence of a second GAG-binding epitope composed of residues K44, K77, K81, K173, and K176 (Site 2; Figure 3.5). This site is located on the opposite side of the helices to Site 1 and is not found in B31 or N40 DBPAs.

3.3.2 GAGs’ Interaction with WT N40, B31 and PBr DBPAs

Previous studies by Leong and co-workers [19, 26] have provided comprehensive analyses of the differential binding affinities of the DBPA variants. Specifically, PBr DBPA has the highest affinity for both decorin and DS; whereas, B31 DBPA shows a slightly weaker affinity and N40 has the weakest affinity for these ligands. We also carried out a qualitative comparison of DBPA-heparin dp6 interactions among the three strains with GMSA, an experiment based on the principle that the migration of heparin dp6 in electrophoresis will be impeded upon binding to DBPA. Figure 3.6 shows the results of the assay.

**Figure 3.6.** GMSA of heparin dp6 in the presence of increasing concentrations of B31 WT, N40 WT, and PBr WT. Both PBr and B31 DBPAs shifted significantly more heparin dp6 than N40 DBPA.

After the addition of one molar equivalent of protein to fluorescently-labeled heparin dp6, both B31 and PBr DBPAs produced shifts in the positions of a significant fraction of
heparin dp6 fragments. In contrast, N40 DBPA induced shifts in a much smaller fraction of fraction of heparin dp6, indicating GAG affinity of N40 DBPA is lower than that of B31 and PBr DBPAs. These results are consistent with previous studies \([19, 26]\).

In order to verify the involvement of the proposed GAG-binding epitopes in binding GAGs, WT PBr’s interaction with heparin dp6 was examined via NMR-monitored titration. Although DS is the GAG-type most commonly found on decorin, our choice of GAG ligands was limited by the fact that solution NMR studies cannot be conducted using natural polysaccharides because of the protein aggregation induced by the long polymers. As a result, only short GAG ligands can be used in these titrations. However, the use of short ligands diminishes the avidity effect that protein-GAG interactions rely on to achieve high affinity. Our previous studies have shown that DBPA’s affinity for DS dp6 is very low (K_D > 10 mM), thus not ideal for identifying GAG-binding epitopes \([21, 60]\). To improve the binding affinity, we chose to use heparin dp6 as our ligand. We have used these ligands previously \([21, 60]\) and DBPAs’ relative affinities for these ligands correlate well with their affinities for intact DS in our experience.

In the titrations of both N40 and PBr DBPAs with heparin dp6, significant changes in the chemical shifts of amide protons and nitrogens were seen (Figure 3.7).
Figure 3.7. Titrations of WT N40 and PBr DBPAs with heparin dp6. (A) $^{15}$N-HSQC overlays of WT N40 DBPA in the presence of increasing concentrations of heparin dp6. The blue contour represents the initial HSQC spectrum of N40 in the absence of heparin dp6. Each subsequent colored contour represents the HSQC spectrum of N40 at the following concentrations of heparin dp6: 0.75, 1.50, 2.25, 3.00, and 3.75 mM. The concentration of N40 was 0.15 mM. Residues experiencing a large migration are indicated with the residue number and the direction of migration. Normalized chemical shift changes for the residues are shown on the left. The residues perturbed the most by the addition of heparin dp6 are notated on each plot. The secondary structure for each protein is displayed at the top of each plot. (B) Titration of 0.15 mM WT PBr DBPA with HSQC spectrum of PBr at the following concentrations of heparin dp6: 0.3, 0.6, 0.9, 1.2, 1.5, and 2.1 mM.

However, N40 DBPA required more ligands to produce shifts of similar magnitudes as PBr DBPA. Because magnitudes of chemical shift changes are a good indication of involvement in ligand binding, these values are often used to identify residues in the GAG-binding sites. Figure 3.7 shows the residue-specific changes in amide proton and nitrogen chemical shift changes for both N40 and PBr DBPAs. Residues N68, T90, and C191 in N40 DBPA produced the biggest changes in chemical shifts. Residues E57, L76, K130, L175, E177, and A179 in PBr DBPA showed the most changes in chemical shifts.
upon the addition of heparin dp6. These residues are found in helix two, the linker between helices three and four, as well as the C-terminus (Figure 3.7B). Most of which are located near either Site 1 or Site 2. It should also be noted that none of the residues in the linker between helices 1 and 2 of PBr showed large changes in their chemical shifts. This is significantly different from what has been observed in B31 DBPA [27].

Besides identifying possible GAG-binding epitopes, we also obtained the $K_D$s of interaction for these proteins by measuring the magnitudes of chemical shift changes induced by different concentrations of heparin dp6. The $K_D$ for N40 DBPA’s interactions with heparin dp6 was calculated to be greater than 4 mM for most residues with large chemical shift changes (Figure 3.8).

**Figure 3.8.** $K_D$ curves of three residues for WT N40. These residues (A56, T90, and Y175) experienced the greatest linear peak migration when titrated with heparin dp6 and were analyzed to give $K_D$ for WT N40.

In contrast, $K_D$ of interaction for B31 DBPA and heparin dp6 is ~0.5 mM [60]. Heparin dp6-binding curves of PBr DBPA residues differed significantly and did not produce a consensus $K_D$ value. For residues near Site 1 (T64, K130, G133), the binding curve showed the site was not saturated at high heparin dp6 concentrations (Figure 3.9), indicating the GAG affinity of the site is low.
Figure 3.9. Titration curves of six residues for WT PBr. These residues (A49, T64, K130, G133, A179, and A182) experienced the greatest linear peak migration when titrated with heparin dp6.

However, another group of residues at the C-terminus (A179 and A182), near Site 2, produced binding curves that showed much higher affinity for GAGs and quick saturation (Figure 3.9). Fitting binding curves independently and assuming that the concentration of the free ligand is not affected significantly by the presence of the other binding site, which is valid because the ligand is present in much higher excess than the protein throughout the titration, we obtained a $K_D$ of $\sim 1.2$ mM for Site 1 residues and $\sim 0.4$ mM for Site 2 residues.

To estimate GAG-binding induced changes in conformational dynamics of the proteins, we used the Model-Free approach [54, 55, 60] to measure the flexibility of the protein backbone in the presence and absence of the GAG ligands. The Model-Free approach combines NMR observables such as longitudinal relaxation rates, transverse relaxation rates and steady state heteronuclear NOE of the atom to derive a parameter known as the order parameter or $S^2$. Values of $S^2$ range from values 0 to 1, with 1 indicating the atom is in a region of the protein having a rigid conformation and 0
indicating the atom is in a completely flexible segment of the protein. We measured the $S^2$ values of backbone amide nitrogen atoms in the presence and absence of the heparin dp6 ligand for both N40 and PBr DBPAs. No large changes in $S^2$ were observed in either protein, indicating that backbone dynamics remain unaffected by GAG-binding (Figure 3.10).

**Figure 3.10.** Order parameters of backbone amide nitrogen atoms for (A) WT N40 in the absence (black) and presence (red) of 24 molar equivalents of heparin dp6 and for (B) WT PBr in the absence (black) and presence (red) of 20 molar equivalents of heparin dp6. Because data for PBr DBPA were collected on a sample with higher concentration that N40 DBPA, the quality of the data was significantly better than that of N40 DBPA, resulting in the error of $S^2$ for PBr being considerably smaller than that of N40.

Although chemical shift mapping is widely used to define ligand-binding epitopes, other phenomena in the protein, such as ligand-induced conformation changes, can produce artifacts that may be misleading. To unambiguously define the GAG-binding epitope, we also probed the protein with heparin dp6 ligands functionalized at the reducing end with the nitrooxide radical TEMPO [60]. The paramagnetic center of TEMPO generates an inhomogeneous magnetic field that causes NMR signals of nearby atoms to experience larger relaxation rates; this phenomenon is termed PRE. PRE is distance-dependent so residues closest to the TEMPO tag will experience the greatest increases in relaxation rates. By measuring the paramagnetic relaxation contribution to
the transverse relaxation rates of amide protons, the location of the reducing end of the bound ligand can be determined with high sensitivity. Both N40 and PBr DBPAs were probed with TEMPO-labeled heparin dp6 and the PRE effect on each residue was quantified by measuring changes in transverse relaxation rates of amide protons after the TEMPO radical was reduced by ascorbic acid. Due to the weak interactions of N40 DBPA with GAGs, the PRE effect was not significant even at a protein-to-ligand ratio of 6 (Figure 3.11).

![Graph showing residue-specific PRE effect](image)

**Figure 3.11.** Comparison of the residue-specific PRE effect on backbone amide protons of WT N40 from TEMPO-labeled heparin dp6 before and after reduction of the TEMPO radical. The comparison indicated that the PRE effect was not significant for any residue.

On the other hand, PBr DBPA experienced significant perturbations after addition of only two molar equivalents of the TEMPO-labeled heparin dp6 ligand. Figure 3.12 shows the residue-specific PRE of each backbone amide proton in PBr.
Figure 3.12. PRE perturbation by TEMPO-labeled heparin dp6. (A) Residue-specific PRE on backbone amide protons of WT PBr from TEMPO-labeled heparin dp6. Residues on helices 1 and 2 (K46, A49, E73, and K75) experienced larger PRE effects when probed with TEMPO-heparin dp6. (B) Ribbon diagram of WT PBr DBPA with the residues experiencing the greatest PRE effect colored in red.

The residues that showed the highest PRE were found near Site 1 (I45, K46, A49, and K79) and Site 2 (E73). These residues are concentrated on helices one and two, indicating that the reducing end of heparin was near these helices. In contrast, the same ligand only produced a strong PRE effect in the linker residues (N59, F60) of B31 DBPA. Besides residues in helices one and two of PBr DBPA, A144 in the short linker between helices four and five also showed a large PRE effect that could indicate either the existence of another weak GAG-binding epitope or non-specific interactions.

3.3.3 Interactions of PBr DBPA Mutants with GAGs

The structure of PBr DBPA and the ligand-binding perturbation analyses suggest PBr DBPA most probably possesses two GAG-binding sites. Site 1 is located at the same location as the major GAG-binding epitopes of B31 and N40 DBPAs. However, another
cluster of basic amino acids on the other side of helices two and five from Site 1 can also be found. This cluster is made up of residues K44, K77, K81, K173, and K176. To determine the contribution of each site to GAG-binding, mutants of PBr DBPA missing either Site 1 or Site 2 basic amino acid clusters were created. In the Site 1 mutant, K79, K130 and K160, three basic amino acids that were found in the pocket and showed large changes in either chemical shift mapping analysis or PRE perturbation, were mutated to serine. In Site 2, residues K77, K81, K173 and K176 were mutated to serine. Although these residues did not show large chemical shift perturbations themselves, residues around them (L76, L175 and E177) did experience either large chemical shift migrations or TEMPO-induced PRE effects. To further dissect the contributions of each basic amino acid pair in Site 2 to GAG-binding, Site 2A (K77, K81) and Site 2B (K173, K176) mutants were also created. GAG-binding affinities of the mutants were evaluated using NMR titrations, GMSA and ELISA. In NMR titrations, each mutant was titrated with heparin dp6 in the same manner as WT PBr to determine the effect of mutations on $K_D$ binding. Table 3.2 lists the apparent heparin dp6 interaction $K_D$s of these mutants.

<table>
<thead>
<tr>
<th></th>
<th>PBr DBPA</th>
<th>$K_D$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Site 1 (K130)</td>
</tr>
<tr>
<td>WT</td>
<td></td>
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</tr>
<tr>
<td>Site 1 Mutant</td>
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<td>--</td>
</tr>
<tr>
<td>Site 2 Mutant</td>
<td></td>
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</tr>
<tr>
<td>Site 2a Mutant</td>
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<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Site 2b Mutant</td>
<td></td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

As expected, mutations in PBr DBPA’s Site 1 significantly reduced the chemical shift changes of residues around Site 1 (A49, T64 and G133) after addition of heparin dp6 (Figure 3.13).
Figure 3.13. $^{15}$N-edited HSQCs of titrations of PBr Site 1 (A), Site 2 (B), Site 2a (C) and Site 2b (D) mutants. Each subsequent colored contour after the blue contour (absence of heparin dp6) represents the HSQC spectrum of the PBr mutant at the following concentrations of heparin dp6: 0.3, 0.6, 0.9, 1.2, 1.5, and 2.1 mM. The concentration of each mutant was 0.15 mM.

However, the apparent $K_D$ estimated from residues near Site 2 is still approximately 0.1 mM, similar to WT PBr (Figure 3.14).
Figure 3.14. $K_D$ curves of six residues for PBr Site 1, PBr Site 2, PBr Site 2a, and PBr Site 2b mutants. These residues (A49, T64, K130, G133, A179, and A182) experienced the greatest linear peak migration when titrated with heparin dp6 and were analyzed to give $K_D$.

Likewise, mutations of residues K77, K81, K173 and K176 in Site 2 to serine scarcely changed $K_D$s of Site 1 residues, but greatly reduced heparin-induced chemical shift.
changes of Site 2 residues. In particular, $K_D$s derived from chemical shift changes of T64, K130 and G133, residues found close to Site 1, decreased from $> 1$ mM to $\sim 0.5$ mM (Table 3.2, Figure 3.14). These observations indicate that both sites are involved in binding GAGs but act independently so that when one site is removed, the other site retains its GAG affinity. To confirm that K77 and K81 acted synergistically with K173 and K176 in binding GAGs, Site 2a mutants were titrated with heparin dp6 and significant decreases in heparin-induced chemical shift changes in C-terminal residues A179 and A182 were observed and $K_D$s estimated using these residues increased from $\sim 0.4$ to 0.8 mM (Table 3.2, Figure 3.14). Mutations of K173 and K176 to serine (Site 2b mutant) completely eliminated chemical shift changes of A179 and A182 (Figure 3.13).

To estimate the contributions of each epitope to PBr DBPA’s GAG-binding activity, GMSA and ELISA were carried out on WT PBr and the mutants at physiological pH. Heparin dp8 GMSA showed that, after addition of one molar equivalent of protein, a significant fraction of heparin dp8 fragments is bound to WT PBr DBPA; whereas, very little of the fragments are bound to the Site 1, full Site 2 and Site 2b mutants, but the Site 2a mutant showed reduced, but consistent, shifting of heparin dp8 fragments (Figure 3.15). These results indicate both sites contribute significantly to GAG-binding by PBr DBPA. However, residues K173 and K176 of Site 2 are more important in promoting GAG binding than K77 and K81.
Figure 3.15. GMSA of heparin dp8 in the presence of increasing concentrations of WT PBr DBPA, Site 1 mutant, Site 2 mutant, Site 2a mutant, and Site 2b mutant.

The GMSA results are also consistent with the heparin ELISA assays in which surface-bound native heparin polysaccharides were probed with His-tagged WT PBr DBPA and mutants. ELISA assays showed that the four mutants bound to heparin with much lower efficiency than WT PBr DBPA (Figure 3.16).

Figure 3.16. Effect of mutations in PBr DBPA on GAG-binding as determined by ELISA. Mutations in either GAG-binding epitope reduced PBr DBPA’s affinity for GAGs.
This includes the Site 2a mutant, which showed significant affinity for heparin dp8 in the GMSA. The discrepancy that the Site 2a mutant showed in GMSA and ELISA is most probably due to methodology differences between GMSA and ELISA. In particular, the rate of dissociation of the GAG-Site 2a mutant complex may be significantly higher than between GAG and WT PBr such that the majority of Site 2a mutants are actually washed off during ELISA. On the other hand, absorbance values of wells coated with mutant PBr DBPAs are slightly higher than the control, implying that mutations of a single GAG-binding site in PBr DBPA did not completely eliminate PBr DBPA’s affinity for intact heparin.

We also performed competitive heparin-binding ELISA between N40 and PBr DBPAs using biotinylated N40 DBPA as a reporter. Our competition assay shows that all PBr DBPA variants, including mutants with the weakest GAG affinities, were tested (Figure 3.17).
Figure 3.17. Effect of PBr DBPA on binding of biotinylated N40 DBPA to heparin-coated microwell plates. Each well contained 4 µg of biotinylated N40 DBPA and either 0, 2, 4, or 6 µg of PBr DBPA variants. All PBr variants easily competed off biotinylated N40 DBPA.

Furthermore, competition assay performed using His-tagged PBr DBPA variants as the reporter revealed that N40 DBPA was not able to significantly reduce the amount of heparin-bound PBr DBPA variants even when the N40 DBPA concentration is twice as high as PBr DBPAs (Figure 3.18).
Figure 3.18. Competitive heparin-binding ELISA using His-tagged PBr DBPA as the reporter. The competitive assay was performed by adding 100 µL of PBS containing 4 µg of His-tagged PBr DBPA variant and either 0, 2, 4, or 8 µg of WT N40 DBPA to each well of heparin-coated microwell plate, and incubated at room temperature for 1 hour. The plate was then incubated with anti-His HRP (Sigma) for 1 hour, and the amount of bound PBr DBPA variant was determined using TMB as the substrate and the reaction was stopped by the addition of 0.1 M HCl.

This shows PBr DBPA’s affinity for heparin is considerably higher than N40 DBPA, such that loss of one of its GAG-binding sites still left the protein with higher GAG affinity than WT N40 DBPA.

3.4 Discussion

Because of the important role DBPA plays in establishing early *Borrelia* spirochetes’ colonization [24], understanding how sequence variability in the protein influences its specificity and affinity for GAGs is crucial to providing a rational explanation for differing pathogenicities of different strains of *Borrelia* spirochetes.
Although there have been many structural studies conducted on DBPAs from strains B31 and N40 of *B. burgdorferi*, other strains, such as PBr, have not been well-studied, making it difficult to rationalize variations in their biological activities. Structural analysis and characterization of GAG interactions for N40 and PBr DBPAs carried out in the present study explain how PBr DBPA’s unique structural characteristics allow it to bind GAGs with greater affinity than B31 and N40 DBPAs. In particular, both B31 and N40 DBPAs have a disulfide bond that tethers the C-terminal tail to helix five. In contrast, PBr DBPA is devoid of both cysteine residues required to form this disulfide bond. As a result, its C-terminus is placed away from Site 1, allowing the formation of the secondary GAG-binding site by placing residues K173 and K176 on helix five close to residues K77 and K81 on helix two. This site could not have formed without the alternate orientation of the C-terminus.

Besides the differing positions of the C-termini, the linker between helices one and two in PBr DBPA is also more structured than in B31 and N40 DBPAs. The significance of this difference lies in the fact that the flexible, unstructured linker found in B31 and N40 DBPAs are positioned directly above Site 1 and may hinder GAGs from accessing the GAG-binding pocket. PBr DBPA’s more compact linker leaves more of Site 1 exposed, giving GAG ligands more access to Site 1. This observation is compatible with the TEMPO-labeled heparin dp6 perturbation data for PBr DBPA which showed that the reducing end of heparin dp6 interacts with residues on helices one and two. This indicates that the fragment was able to enter the pocket. In comparison, linker residues in B31 DBPA were the ones affected most significantly by PRE [60]; whereas, there were very little PRE effects on N40 DBPA residues. Finally, the apparent heparin dp6 K_D of
PBr’s Site 1, in the absence of Site 2, is much higher than that of N40 DBPA (0.4 mM compared with > 4 mM), even though distributions of basic amino acids in both pockets are similar (Figure 3.19).

**Figure 3.19.** Conservations of basic amino acids in the primary GAG-binding pocket of N40 (green) and PBr (tan) DBPA. All basic amino acids contained in the basic pocket of PBr DBPA are conserved in N40 DBPA. In addition, N40 DBPA, also has two extra basic amino acids (K139 and R169) which are not in PBr DBPA.

These data show that the obstruction of the pocket may be an important factor in determining the GAG-affinity of Site 1. This model of DBPA-GAG interaction also explains why the basic amino acid cluster (the BXBB motif) in the linker of B31 DBPA is important to the protein’s affinity for GAGs; it compensates for the reduced access to
the binding pocket by providing additional binding epitopes for GAGs, thereby increasing B31 DBPA’s affinity for GAGs. In contrast, N40 is missing both Site 2 and the basic amino acid cluster in its linker, resulting in a much lower affinity for GAGs despite the fact that the number of basic amino acids in N40 DBPA is almost identical with B31 DBPA (28 basic amino acids in N40 DBPA compared with 29 basic amino acids in B31). Whereas PBr does not contain the basic amino acid cluster in its linker, PBr DBPA compensates for this by having a second GAG-binding site and a retracted linker that allows more access to Site 1.

In addition, titrations of PBr DBPA mutants with heparin dp6 showed that the two sites are independent of each other. This independence is manifested in the fact that elimination of either site has not effect on GAG affinity of the remaining site. In fact, the apparent $K_D$ of binding estimated from several residues close to Site 1 decreased significantly after mutations in Site 2, implying Site 1’s affinity for GAGs increased with elimination of Site 2. Interestingly, the magnitudes of decreases were larger than decreases predicted with the assumption that Site 2 only binds a single ligand. Specifically, because the ligand concentration is much higher than the protein concentration, an active Site 2 should not change the concentration of free ligand significantly if Site 2 only binds a single ligand. One possible explanation for the unexpected large changes in Site 1 $K_D$ is that Site 2 is capable of simultaneously binding more than one ligand, and thus can bind a higher amount of GAG fragments than expected. Its elimination would increase the concentration of free GAG ligand more than expected. Another possibility is that mutations of Site 2 produced significant changes in the structure of PBr DBPA, leading
to enhanced ligand affinity in Site 1. However, this hypothesis is not supported by the fact that the $^{15}$N-edited HSQCs of both Site 2 mutants are very similar to WT PBr DBPA (Figure 3.13), indicating the structures have not changed significantly in these mutants. Even though an accurate $K_D$ of interaction is not known for Site 1 of PBr DBPA, the dramatic decreases in PBr DBPA mutants’ affinities for heparin in ELISA indicate both sites are important for PBr DBPA under physiological conditions. It also highlights the importance of the multivalent effect on protein-GAG interactions. Multivalency will be especially critical *in vivo* where native GAG chains are capable of binding multiple epitopes simultaneously. These structures of PBr, B31 and N40 DBPAs show that despite having similar topologies, subtle changes in sequences can result in significant structural variations that contribute greatly to differences in the adhesion activities of the protein. Characterization of their structures and GAG interactions can therefore provide crucial insights into how strain variations may contribute to the pathogenicity and offer hypotheses for further *in vivo* experimentation.
CHAPTER 4

Flexible Linker Modulates Glycosaminoglycan Affinity of Decorin Binding Protein A

4.1 Introduction

So far, structure of DBPA from three different strains of *Borrelia* spirochetes have been determined [21, 29, 60]. Despite the sequence differences, their structures are very similar. All structures contain five helices and two flexible segments. The helices form a bundle that is stabilized by a considerable hydrophobic core, and a GAG-binding epitope can be found on its surface (Figure 4.1).

![Figure 4.1. Ribbon representations of B31, N40, and PBr DBPA structures. Basic amino acids found in the GAG-binding pocket are labeled. The extra cluster of basic amino acids found in the linker of B31 is colored red. The disulfide bond found tethering the C-terminus to helix 5 in B31 and N40 is colored yellow.

This GAG-binding epitope includes at least four basic residues from helices one, two and five, and their assembly produces a sizable basic pocket. Mutagenesis studies showed that this basic pocket is critical to DBPA’s affinity for GAGs [29, 50, 60]. Intriguingly, the basic pocket is proximate to a long unstructured linker between helices one and two as
well as an unstructured C-terminus. A NMR dynamics study shows both segments are highly flexible [21], and the crystal structure also showed high B factors indicating that these residues are dynamic [29]. Moreover, both segments remained flexible even in the presence of GAGs [21, 69]. Despite their flexibilities, both unstructured segments seem to play a role in GAG binding. A previous structural study of DBPA from strain B31 showed residues in these two segments experienced higher amide proton and nitrogen chemical shift perturbations upon binding heparin than residues in the basic pocket [60]. This indicates the flexible segments, far from being innocuous bystanders, may play a significant role in modulating GAG binding.

In principle, potential roles of the linker could be gleaned by comparing DBPA structures of B31 and N40 strains. Despite ~80% sequence identity and identical basic amino acid arrangement in the binding pocket (Figure 4.1), the two DBPAs have significantly different affinities for GAGs [19, 26]. A mutagenesis study showed extra basic amino acids in the linker of B31 DBPA (K64, K66 and K67) are a contributing factor to the differences in their GAG affinities. In particular, mutating these basic amino acids to serine significantly reduced GAG affinity, indicating the amino acid composition of the linker is a critical factor in determining GAG affinities. However, DBPA from the PBr strain also lacks basic amino acids in its linker, and yet this version of DBPA has the highest GAG affinity of all strains studied by Leong and co-workers [19, 26]. Comparative structural analysis showed that PBr DBPA has a structured linker between helices one and two, leading to the retraction of the linker (Figure 4.1). These observations led us to propose that the linker of DBPA serves, not as a facilitator of GAG
binding, but actually impedes interactions by blocking access to the basic pocket, therefore explaining why PBr DBPA has much higher GAG affinity.

In this study, we report the experimental evidence to support this hypothesis. Specifically, we show that N40 DBPA mutants with shortened linkers have significantly higher GAG affinities. Furthermore, non-specific surface perturbations and paramagnetic-tagged GAG ligands revealed the GAG-binding pocket of N40 DBPA mutants with shortened linkers is more accessible to GAG than the WT, consistent with the theory that accessibility of the GAG-binding pocket can be improved with the removal of the linker. This mechanism of GAG affinity modulation explains much of the known relationship between structural differences and GAG affinities of DBPAs from various *Borrelia* spirochete strains. To our knowledge, this is the first report of GAG affinities being modulated by dynamic segments outside the binding epitope. It also shows the number of basic amino acids in GAG-binding epitopes may not be the only determining factor of proteins’ GAG affinities.

4.2. Experimental Procedures

4.2.1. Expression and Purification of N40 DBPA Variants

ORF of N40 DBPA was synthesized by GenScript Inc. (Piscataway, NJ). The ORF for the WT mature DBPA strain N40 (residues 29-194) was cloned into the pHUE vector, resulting in a fusion protein of His-tagged ubiquitin at the N-terminus [42]. The mutants for N40 DBPA were created using site-directed mutagenesis from the WT sequence for DBPA (only N40Δ62-71 neat mutant used N40Δ62-71 sequence). The forward primers for the DBPA linker deletion mutants are the following: N40Δ62-66, 5’-ATCAAAAGGATGCGGCCGACAACAATGTCACCAGATTCCGAAACGGGCGAGT-3’.
AAAGTG-3'; N40Δ62-71, 5'-

ATCAAAAAAGGATGCGGCCGACAAACAATGTCGCCAGTAAAGTGGCTCCGAAAAC
TCATTT-3'; and N40Δ62-74, 5'-

ATCAAAAAAGGATGCGGCCGACAAACAATGTCGGAAGAAGGCGCTCATTTATCC
TGGAAGCA-3'. The forward primers for the neutral linker mutants are the following:

D68S/E70S, 5'-GCAGCTTTTCACCAGCTCCACGCGGCAGTAAAGTGGCCG-3',
K74S (the mutations D68S/E70S/K74S are for the mutant N40neutral), 5'-

GCTCCACGACGCCAGTAGCGGTCCCCGAAAAACTCG-3', and N40Δ62-71 K74S
(N40Δ62-71, neu), 5'-GCCGACAAACAATGTCGCCAGTAGCTGCTCCGAAAACCTC-3'.
The forward primers for the DBPA C-terminal mutant (C176,191S) to remove the
disulfide bond are the following: C176S, 5'-

GTCCACACGAAAAACTACACGCGACTGGAAAAGAAAAACCCG-3' and
C191S, 5'-

CCGAATTTACCAGCAGAAAAAGGCAAAAAACAATTAAAAGCTTAGATCCG-3'. Sequencing was used to confirm the correct deletion mutations.

Each plasmid was transformed into E. coli BL21(DE3); then, the bacteria were grown at
37°C to an OD600 of 0.5. Isotopically labeled protein was grown in M9 medium
supplemented with 15NH4Cl and glucose. The bacteria were induced with 0.5 mM IPTG
and incubated overnight at 30°C. To lyse the cells after harvesting, the resuspended pellet
was incubated with 1 mg/mL lysozyme and sonicated. The fusion protein was extracted
from the cleared supernatant via Ni affinity chromatography with a 5 mL HisTrap
column (GE Life Sciences) and was eluted using an imidazole gradient of 25 mM to 300
mM at a flow rate of 3 mL/min. The collected fusion protein was exchanged into 25 mM
Tris (pH 8.0), 100 mM NaCl and was digested with USP2 as well as 1 mM DTT overnight at room temperature. A small volume of His-tagged protein was not digested for use in ELISA. The cleaved DBPA was separated using a 5 mL HisTrap column and was eluted in the flow-through that was then collected, concentrated, and exchanged into 50 mM phosphate pH 6.5, 150 mM NaCl.

4.2.2 ELISA Analysis of Heparin and DS Binding to N40 DBPA Variants

Intact heparin and DS were biotinylated and quantified with the Biotinylation Quantitation kit (Pierce) according to manufacturer’s instructions. Neutravidin-coated microplates (G-Biosciences) were coated with 100 µL of PBS containing 20 µg/mL of biotinylated heparin or DS and washed before being incubated with 2 µg of His-tagged WT N40 DBPA and N40 DBPA mutants in 100 µL PBS for one hour. The bound protein was quantified using 1:2000 Anti-His HRP (Qiagen) and developed with TMB substrate solution. Finally, 100 µL of 0.1 M sulfuric acid was added to each well to stop the reaction after two minutes (heparin) or after twelve minutes (DS), inducing a color change that was read at 450 nm. Each ELISA assay was performed at least twice. Each time, data from four replicates of every sample were averaged to obtain the mean and standard deviation.

4.2.3 Production of Heparin and DS Fragments

Commercially available heparin and DS were digested with either heparinase I (heparin) or Chondroitinase ABC (DS) until 30% of available sites were cleaved. Digests were monitored by measuring absorbance at 232 nm to determine the extent of the digests. The partially depolymerized GAGs were then separated with a size exclusion chromatography column (Bio-Rad Biogel P10) to obtain homogeneous size-defined
fragments. Through reductive amination, the reducing end of heparin dp6 was labeled with 4-amino-TEMPO according to the method of Morgan et al. [60]. Briefly, 400 µL of 5 mg/mL GAG fragments were reductively aminated with 0.3 M 4-amino-TEMPO using 20 mM NaCNBH₃ as the reducing agent. To prevent reduction of the radical, the reaction was carried out in H₂O at neutral pH and 65°C overnight.

4.2.4 Gel Mobility Shift Assays for N40 DBPA Variants

Heparin dp8 and DS dp8 fragments were fluorescently labeled with 0.1 M 2-AMAC as described by Lyon et al. [32]. The assay was performed by incubating 2 µg of the 2-AMAC labeled heparin dp8 or DS dp8 with either one-half (heparin) or one (DS) molar equivalents of DBPA in 50 mM sodium phosphate pH 6.5, 150 mM NaCl for a total volume of 12.5 µL. The reaction mixtures were incubated at room temperature for 30 minutes and were run in a 2% agarose gel at 120 V for 12-20 minutes. A UV panel was used to visualize the shifts [44]. Quantification of the amount of fragments shifted was carried out by comparing brightness-weighted pixel counts of the free bands in the presence and absence of the protein.

4.2.5 Titrations of WT and Mutant N40 with Heparin dp8

¹H—¹⁵N HSQC spectra were collected on a Bruker Ultra-Shield 600 MHz spectrometer. The NMR titration data were used to estimate Kᵥ values for the interaction between heparin dp8 and the DBPA N40 variants at pH 6.5. A total of 18 molar equivalents of heparin dp8 were added in aliquots of either two or four molar equivalents to 400 µL of 150 µM protein. The chemical shift changes noted in each ¹H—¹⁵N HSQC were normalized into one chemical shift value using Equation 1.1 [35]. The binding
curves were fitted using the one-to-one binding model contained in the software xcrvfit (http://www.bionmr.ualberta.ca/bds/software/xcrvfit/) to extract the $K_D$ values.

4.2.6 Acquisition and Analysis of PRE

NMR data for PRE experiments were collected on Bruker Ultra-Shield 600 MHz spectrometer. To detect specific N40-GAG interaction using paramagnetic heparin dp6 ligands, two molar equivalents of TEMPO-tagged heparin dp6 were added to 400 µL of 200 µM protein (N40 WT and N40Δ62-71). To detect surface exposed residues, 20 mM 4-hydroxy-TEMPO or 4-amino-TEMPO were added to 350 µL of 200 µM protein (N40 WT and N40Δ62-71). The paramagnetic contribution to the transverse relaxation was measured as the difference between $^1$H $T_2$ before and after reduction of the TEMPO radical with either ten molar equivalents (TEMPO-tagged heparin dp6) or two molar equivalents (4-hydroxy-TEMPO or 4-amino-TEMPO) of ascorbate [62].

4.2.7 ITC

(ITC was conducted by Krishna Sepuru and Krishna Rajarathnam, Department of Biochemistry and Molecular Biology, Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA)

The binding affinities of DBPA N40 variants were characterized by measuring the heat changes on titrating dp10 heparin into the DBPA solution using a Microcal VP Titration Calorimeter. Protein and GAG solutions were dissolved in 50 mM phosphate 150 mM NaCl (pH 6.5), centrifuged, and degassed under vacuum before use. Titrations were performed by injecting 2 x 2 µL and 6 x 46 µL aliquots of GAG into DBPA solution at 25°C. Data were analyzed using Origin software supplied by Microcal.
4.3 Results

To probe the role of the linker in modulating GAG affinity of DBPA, we constructed several mutants containing truncated linkers. We used WT N40 DBPA as our model because, unlike B31 and PBr DBPAs, its linker is not known to contain elements that enhance GAG binding. Chemical shift perturbation analysis and paramagnetic perturbation using TEMPO-labeled heparin dp6 ligands both showed the linker of N40 DBPA is minimally perturbed by heparin [69], indicating its interactions with GAGs are most likely minimal. This property of N40 DBPA allows us to objectively evaluate the impact of the linker length on GAG affinity without the complication of removing additional epitopes in the process.

Figure 4.2 shows the mutants that were constructed for this study.

![Sequence of helical regions of WT N40 DBPA are highlighted in black. The mutations made in this study are indicated as follows: the three linker deletion mutations are underlined, the neutral linker mutations are shaded gray and the disulfide bond mutation is notated with a (*). The linker deletion mutants are the following: N40Δ62-66, N40Δ62-71, and N40Δ62-74. The neutral linker mutants include N40neutral and N40Δ62-71, neu. The C-terminal disulfide bond was removed by the mutant C176S/C191S. Specifically, we prepared three N40 DBPA mutants with five (residues 62 to 66), ten (residues 62 to 71) and thirteen (residues 62 to 74) residues removed from the linker. These are designated as N40Δ62-66, N40Δ62-71 and N40Δ62-74, respectively. Residues were chosen after the impact of linker shortening on nearby secondary structures were modeled using the program, modeller [70], to ensure the integrity of the helices are preserved.]
integrity of helices one and two flanking the linker was also verified for the mutant N40\(_{\Delta62-71}\) using NMR. In particular, we observed existence of sequential HN—HN NOESY cross-peaks characteristic of helices, and backbone dihedral angles predicted from C\(_\alpha\) and C\(_\beta\) chemical shifts are also consistent with the retention of the helical structure (Table 4.1).

**Table 4.1.** Phi and psi angles from TALOS+ [68] for the residues surrounding the N40\(_{\Delta62-71}\) deletion confirm alpha-helical secondary structure.

<table>
<thead>
<tr>
<th>N40 Residue</th>
<th>Phi ((\phi)) Angle</th>
<th>Psi ((\psi)) Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Helix One</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A56</td>
<td>-64° ± 5°</td>
<td>-41° ± 8°</td>
</tr>
<tr>
<td>A57</td>
<td>-62° ± 5°</td>
<td>-40° ± 6°</td>
</tr>
<tr>
<td>D58</td>
<td>-63° ± 8°</td>
<td>-40° ± 7°</td>
</tr>
<tr>
<td>N59</td>
<td>-66° ± 7°</td>
<td>-35° ± 12°</td>
</tr>
<tr>
<td>N60</td>
<td>-101° ± 19°</td>
<td>3° ± 21°</td>
</tr>
<tr>
<td><strong>Linker</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V61</td>
<td>-75° ± 55°</td>
<td>129° ± 14°</td>
</tr>
<tr>
<td>G72</td>
<td>89° ± 8°</td>
<td>2° ± 15°</td>
</tr>
<tr>
<td>S73</td>
<td>-62° ± 71°</td>
<td>115° ± 54°</td>
</tr>
<tr>
<td>K74</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>V75</td>
<td>-96° ± 22°</td>
<td>-6° ± 22°</td>
</tr>
<tr>
<td>S76</td>
<td>-90° ± 62°</td>
<td>145° ± 24°</td>
</tr>
<tr>
<td>E77</td>
<td>-77° ± 60°</td>
<td>124° ± 26°</td>
</tr>
<tr>
<td>N78</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Helix Two</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S79</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>F80</td>
<td>-63° ± 6°</td>
<td>-42° ± 7°</td>
</tr>
<tr>
<td>I81</td>
<td>-63° ± 5°</td>
<td>-63° ± 9°</td>
</tr>
<tr>
<td>L82</td>
<td>-63° ± 6°</td>
<td>-36° ± 8°</td>
</tr>
<tr>
<td>E83</td>
<td>-63° ± 5°</td>
<td>-42° ± 6°</td>
</tr>
<tr>
<td>A84</td>
<td>-63° ± 5°</td>
<td>-43° ± 5°</td>
</tr>
<tr>
<td>K85</td>
<td>-64° ± 5°</td>
<td>-33° ± 10°</td>
</tr>
</tbody>
</table>

n.a. - no assignment for HN, N, CA, and/or CB

Beside the linker truncation mutants, we also engineered mutants without the charged amino acids in the linker and a mutant missing the disulfide bond connecting the flexible C-terminal linker to helix five. This disulfide bond tethers the flexible C-terminus close to the core domain and the basic pocket. However, the disulfide bond is missing in a
number of DBPA sequences, such as PBr DBPA. The Cys mutant (C176S/C191S) of N40 DBPA will allow us to evaluate whether the disulfide bond has any functional role in DBPA’s GAG-binding activity. There are also a number of charged amino acids in the linker of N40 DBPA (D68, E70 and K74). Removal of these amino acids may potentially influence DBPA-GAG interactions. To gauge their contributions to GAG binding, we also created a mutant having a linker of normal length but with the three charged amino acids mutated to Ser (N40neutral) and another version of the N40Δ62-71 mutant also containing a neutral linker (N40Δ62-71, neu).

We first tested the mutants’ affinities for intact GAGs with ELISA assays that use immobilized heparin and DS as probes. In the case of immobilized heparin, mutants with shortened linkers showed a dramatic increase in heparin binding compared to the WT while the C176S/C191S mutant showed a small decrease in GAG affinity (Figure 4.3). We also performed ELISA using immobilized DS, the major GAG-type found on decorin, as the probe. Similar to heparin, significant improvements in DS-binding affinity can be seen for all linker truncation mutants (Figure 4.3), but the improvements are small than that of heparin.
Figure 4.3. Effect of mutations in N40 DBPA on GAG-binding as determined by ELISA. Shortening the linker increased N40 DBPA’s affinity for GAGs while removal of the C-terminal disulfide bond reduced the affinity. Student’s t test comparing WT N40 DBPA with each mutant indicates the change in GAG-binding affinity is statistically significant ($p < 0.0001$ for both heparin and DS).

These results indicate GAG affinity enhancements stemming from linker shortening may be dependent on GAG type, and this is potentially a factor in determining the preference of DBPA for a specific tissue type. Similar to the heparin ELISA, the C176S/C191S mutant showed a small but statistically significant ($p < 0.01$) decrease in its DS affinity.

We also carried out ELISA on mutants with neutral linkers to measure whether the enhancement in GAG affinity is the result of linker-length shortening or the elimination of the charged amino acids. Figure 4.4 shows the results of these assays.
Specifically, we saw that the mutant with normal linker-length but no charged amino acids in the linker has about the same binding affinity for GAGs as WT N40 DBPA, indicating the net effect of the charged amino acids on GAGs is minimal. Shortening the linker resulted in a substantial increase in both heparin and DS affinities of the protein. Specifically, N40_{Δ62-71, neu}’s affinity of heparin increased ~2.5 fold while its DS affinity doubled. These are comparable to increases seen in N40_{Δ62-71}.

To obtain a more quantitative insight, we also probed the binding interactions using size-defined heparin and DS oligosaccharides. First, GMSA was conducted to qualitatively evaluate the affinities of the mutants for heparin dp8 and DS dp8. The
GMSA measures the extent to which a protein is capable of impeding the electrophoretic migration of fluorescently labeled GAG ligands. The results showed that the linker-truncated N40 DBPA mutants have significantly higher affinity for heparin dp8 than the WT, as demonstrated by the large amount of heparin fragments whose migration the mutants have impeded (Figure 4.5A).

Figure 4.5. GMSA of heparin dp8 (A) and DS dp8 (B) in the presence of either 1 or 0.5 equivalents of WT N40 DBPA, N40Δ62-66, N40Δ62-71, N40Δ62-74, or N40 C176S/C191S mutants.

On the other hand, heparin affinity of the C176S/C191S mutant was no different compared to the WT. The linker mutants also significantly increased the amount of DS dp8 fragments shifted compared to the WT, but the C176S/C191S mutant did not show
significant changes in DS affinity compared to the WT (Figure 4.5B). Both are in agreement with the ELISA results. However, because of DBPA’s weaker affinity for DS, twice as much DBPA was required to shift a comparable amount of DS fragments as heparin fragments.

We then characterized these interactions using solution NMR titrations. We used heparin oligosaccharides as the representative GAG because our previous NMR work has shown that DBPA-binding to DS fragments fall in the intermediate exchange regime on the NMR time scale, preventing accurate extraction of $K_D$ values, and GMSA assays indicate that the relative heparin affinities reliably capture their affinities for DS [21, 60]. For the NMR studies, the mutants were titrated with heparin dp8 fragments, and the binding-induced chemical shift perturbations from basic pocket residues having larger perturbations (T90, T140 and R169) were measured and fitted to obtain the $K_D$. Figure 4.6 shows the chemical changes of T140 in WT and mutant N40 DBPAs as well as the binding curves derived from these data.
Figure 4.6. $^{15}$N-HSQC overlays of residue T140 of N40 DBPA variants in the presence of increasing concentrations of heparin dp8. The lightest contour represents the initial HSQC spectrum of N40 in the absence of heparin dp8. Each subsequent contour represents the increasing concentrations of heparin dp8: 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 2.0 mM. The concentration of N40 was 0.15 mM. Overlay of $K_D$ curves for T140 of each N40 variant is shown in the bottom right panel.
Table 4.2 summarizes the K_Ds of linker truncated DBPAs.

**Table 4.2.** N40 linker mutants show increased binding to heparin fragments (heparin dp8 from NMR and heparin dp10 for ITC).

<table>
<thead>
<tr>
<th>N40 Variant</th>
<th>NMR</th>
<th>ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_D (µM)</td>
<td>K_D (µM)</td>
</tr>
<tr>
<td>WT</td>
<td>&gt; 2 mM</td>
<td>&gt;650</td>
</tr>
<tr>
<td>N40Δ62-66</td>
<td>495 ± 59</td>
<td>617 ± 55</td>
</tr>
<tr>
<td>N40Δ62-71</td>
<td>234 ± 11</td>
<td>153 ± 7</td>
</tr>
<tr>
<td>N40Δ62-74</td>
<td>200 ± 20</td>
<td>219 ± 6</td>
</tr>
<tr>
<td>C176S/C191S</td>
<td>751 ± 176</td>
<td>&gt;650</td>
</tr>
</tbody>
</table>

Shortening the linker decreased K_D from several mM to ~0.2 mM for mutants with linkers shortened by ten residues or more. On the other hand, K_D for the disulfide bond mutant, C176S/C191S N40 DBPA, only decreased from several mM to ~0.8 mM. This is qualitatively consistent with GMSA results, where no difference was seen between WT N40 DBPA and the C176S/C191S mutant. In total, these experiments demonstrate the effect of removing the disulfide bond on N40 DBPA’s affinity for the heparin ligand is small.

We also determined the global binding affinities of the mutants using ITC to independently validate K_D from the NMR experiments. The size of heparin was increased by a disaccharide to obtain a higher change in enthalpy so the K_Ds can be accurately measured. Figure 4.7 and Table 4.2 summarize the result of the ITC experiments. The ITC data show that mutants with shortened linkers were able to produce significantly higher changes in enthalpy than WT or C176S/C191S N40 DBPA.
Figure 4.7. ITC titration curves of N40 DBPAs with heparin dp10. Values of $K_D$'s extracted from these data are shown in Table 4.2.

Trends of $K_D$ changes between the WT and mutants measured are also in complete agreement with GMSA and NMR titration data. Namely, mutants with shortened linkers showed significantly lower $K_D$'s than the WT, and C176S/C191S N40 DBPA showed negligible change compared to the WT.

To verify that the enhancements in GAG affinities of DBPAs are the result of a more accessible basic binding pocket, we probed the GAG-binding sites of both WT
DBPA and N40\textsubscript{Δ62-71} with paramagnetic 4-amino-TEMPO and 4-hydroxy-TEMPO. Paramagnetic tags produce distance-dependent relaxation enhancements in nearby atoms by a phenomenon known as PRE \cite{71}. To obtain the paramagnetic contribution to relaxation, the amide proton transverse relaxation rates in the presence of the paramagnetic form and the ascorbic acid-reduced diamagnetic form of the same ligand were measured. The differences between the two rates, which is due entirely to the presence of a paramagnetic center, is known as R\textsubscript{2,PRE}, the size of which is directly proportional to the inverse of the sixth power of the average distance between the paramagnetic center and the atom. Because of the strong gyromagnetic ratio of the electron, PRE has an effective range of 20 Å if the nitrooxide radical is used as the paramagnetic center and can effect detectable changes in NMR signals even if contacts between the paramagnetic center and the atom is transient. In the case of hydroxy-TEMPO and amino-TEMPO, these paramagnetic reagents are either neutral or positively charged at pH 6.5 and therefore should only have weak or non-specific interactions with the protein surface, making them good indicators of surface accessibility. PRE effects of 20 mM hydroxy-TEMPO on WT N40 and N40\textsubscript{Δ62-71} are shown in Figure 4.8.
Figure 4.8. Surface perturbation PRE on backbone amide protons of WT N40 (top) and N40Δ62-71 (bottom) from 4-hydroxy-TEMPO to detect surface exposed residues. Residues in the mutants’ binding pocket, compared to the WT, experienced larger PRE effects when probed with 4-hydroxy-TEMPO. $R_{2,\text{PRE}}$ values of the residues are mapped onto ribbon diagrams of WT N40 and N40Δ62-71 with a white-to-red gradient that reflects $R_{2,\text{PRE}}$ values of 10 to 72.

For WT N40, amide proton of most residues experienced small $R_{2,\text{PRE}}$ between 5 to 15 s$^{-1}$. However, several residues were severely perturbed. Signals of residues N62, F63 and T172 were broadened beyond detection and only reappeared after the reduction of the radical. Signals from G32 and V125 also experienced severe, but measurable, PRE effects. These strong PRE perturbations are most likely the result of strong non-specific interactions between these residues and hydroxy-TEMPO. Upon shortening of the linker,
the number of residues that disappeared due to PRE effects and reappeared after radical reduction increased from 3 to 16. In particular, signals from residues E33, I52 to K54, A56, A57, G72, S73, F80, L82, K85, E93, V125, L132, G133 and R136 only reappeared after radical reduction while $R_{2,\text{PRE}}$ for residue E83 was measured at $\sim72 \pm 11$ s$^{-1}$. The majority of these residues can be mapped to helices 1 and 2 in the basic pocket. In the case of positively charged amino-TEMPO, the overall PRE perturbation on WT N40 is similar to the case of hydroxy-TEMPO (Figure 4.9). Specifically, signals of N62, F63 and V125 were broadened beyond detections, indicating some non-specific interactions between the protein and the ligand exist.
Figure 4.9. Residue-specific PRE on backbone amide protons of WT N40 (top) and N40Δ62-71 (bottom) from 4-amino-TEMPO to detect surface exposed residues. Residues in the mutant’s binding pocket experienced larger PRE effects than the same residues in the WT when probed with amino-TEMPO. R\textsubscript{2,PRE} values of the residues are mapped onto ribbon diagrams of WT N40 and N40Δ62-71 with a white-to-red gradient that reflects R\textsubscript{2,PRE} values of 10 to 45.

Once again, shortening the linker greatly increased the number of signals broadened beyond detection from 3 to 10 (T46, I52 to K54, A56, A57, K85, V125, L132, G133 and Q147) while G72 and F80 experienced strong, but measurable, PRE effects. These experiments demonstrate that the basic binding pocket is significantly more exposed after linker shortening.
We also probed the binding pockets of WT N40 DBPA and N40Δ62-71 mutant with TEMPO-labeled heparin derivatives (Figure 4.10).

![Residue-specific PRE on backbone amide protons](image)

**Figure 4.10.** Residue-specific PRE on backbone amide protons of WT N40 (top) and N40Δ62-71 (bottom) from TEMPO-tagged heparin dp6. Residues in the mutant’s binding pocket experienced larger PRE effects than the same residues in the WT when probed with TEMPO-heparin dp6. \(R_{2,PRE}\) values of the residues are mapped onto ribbon diagrams of WT N40 and N40Δ62-71 with a white-to-red gradient that reflects \(R_{2,PRE}\) values of 10 to 45.

In the case of WT N40 DBPA, residues that did show perturbation were mostly in the flexible segments such as the linker and the C-terminus. However, the binding pocket basic residues in the N40Δ62-71 mutant showed much higher PRE. In fact, residues K53, K166 and R169, all of which are part of the GAG-binding epitope in the basic pocket,
completely disappeared and only reappeared when the tag was reduced. This behavior is in agreement with the surface perturbation data and shows the basic pocket is also the main GAG-binding site in N40 DBPA.

4.4 Discussion

The results of this study show the flexible linker of DBPA is a critical negative regulator of GAG affinity. This is consistent with the theory that the linker occludes the binding pocket and reduces access of GAGs, thus reducing the protein’s affinity for GAGs despite having a rich collection of basic amino acids in the pocket. Shortening the linker removes the obstruction and increases GAG affinity (Figure 4.11).

![Diagram](image)

**Figure 4.11.** Schematic illustrating the regulatory effect of the flexible linker in three strains of DBPA (B31, N40 and PBr).

This is a novel result because conventional wisdom believes that GAG affinities of GAG-binding proteins are controlled mostly by the number of basic amino acids in the epitope. However, residues that were removed in this study are not part of the epitope and the number of basic amino acids in the pocket was not altered. In addition, ELISA results show that the GAG affinity enhancement gained from shortening the linker does not apply to all GAG types equally. Specifically, linker truncation enhanced heparin binding to a much greater extent than DS binding.
These results explain much of the observed differences in GAG affinities of DBPAs from different strains. N40 DBPA, whose basic pocket constitutes the only major GAG-binding epitope, has the weakest affinity because of lack of access to the basic pocket by the ligand \cite{19, 26}. B31 DBPA, on the other hand, possesses an extra basic amino acid cluster in its linker, allowing for increased electrostatic interactions between the protein and GAGs through this more exposed epitope. PBr DBPA evolved another alternate strategy to overcome the barrier of occluded GAG-binding site by having a helical linker whose compact shape allows the pocket to be much more exposed and accessible for interactions with GAGs. Furthermore, because the extent of the enhancement is GAG-type dependent, PBr DBPA will have a significantly higher affinity for heparin-like GAGs such as HS than N40 or B31 DBPAs. This difference in affinity for heparin-like GAGs may be a factor in determining tissue tropism during infection. Knowing the existence of this mechanism for GAG affinity regulation will therefore allow more accurate prediction of GAG affinities of DBPAs and their effects on localization of Lyme disease.

The fact that the linker residues occlude the basic pocket does not mean the basic residues in the pocket do not play a role in mediating GAG binding. Indeed, previous studies have shown that mutations of these residues to serine reduced GAG affinity by varying degrees \cite{29, 50, 60}. These residues can contribute to GAG binding through at least two mechanisms: first, because electrostatic forces are long-range interactions, the presence of these residues in the vicinity may be sufficient to attract GAGs without direct contact and the protein-GAG complex can then be stabilized through formation of hydrogen bonds with polar residues in the linker; second, the large size of most native
GAG chains means multivalent interactions between DBPA and GAGs will significantly boost DBPA’s affinity for them. It also should not be surprising that evolutionary pressures have not managed to produce a DBPA with a more efficient GAG-binding mechanism. Higher GAG affinity does not imply more efficient colonization. Biological processes such as bacterial infection are usually fine-tuned to achieve an optimal level of interactions to allow adhesion to the host without attenuating proper dissemination. Furthermore, even though these proteins’ affinity for GAGs is weakened, through avidity effects, they may still produce sufficient adherence to prevent the bacterium from being cleared from the host tissue. Other GAG-binding adhesins may also be in place to compensate for DBPAs’ weaker affinity, thus reducing the pressure on the bacterium to generate a more efficient GAG adhesin.

GAG-binding proteins constitute an important class of proteins that control critical biological phenomena such as leukocyte trafficking, blood coagulation and cancer cell metastasis [72, 73]. Because of their importance, there has always been an interest in understanding factors determining these proteins’ specificity and affinity for GAGs. However, high resolution structure information of these systems is scarce because of the dynamic nature of these interactions and heterogeneity existing in the GAG ligands. Available structural data show that GAG-binding epitopes in these proteins adopt diverse conformations and have little sequence homology. Although most of these epitopes contain basic amino acids, the number of basic amino acids in the epitope is not always a good predictor of the binding affinity [5]. Most previous studies on factors regulating GAG affinities have focused on the position and prevalence of basic amino acids. Our current work presented here shows that GAG affinities can also be regulated by linker
domains functioning as flaps. As far as we know, DBPA is the only reported example of a protein regulating GAG affinity using such a mechanism. However, given the prevalence of unstructured loops in GAG-binding proteins, more proteins with such a regulatory mechanism may be identified in the future.
CHAPTER 5

Probing Protein-Glycosaminoglycan Binding Sites with 4-Carboxy-TEMPO and Paramagnetic Relaxation Enhancement

5.1 Introduction

The complexity and structural diversity of GAGs allow them to control many biological processes through their interactions with GAG-binding proteins [1]. Understanding the biophysical mechanism through which these proteins bind GAGs can have important applications because disruption of these interactions have been shown to attenuate function [3, 74, 75]. However, there is little structural or dynamic information regarding these interactions. Of the few known structures of protein-GAG complexes, the majority involve binding to heparin-like oligosaccharides [76]. While many GAG-binding proteins do bind heparin, the similarities in the unmodified GAG backbones often allow the protein to bind more than one type of GAG through electrostatic interactions with its GAG-binding epitope. The lack of structural data on protein-GAG complexes is partly the result of the heterogeneous and dynamic nature of the GAG molecule itself. Structurally homogeneous samples of GAGs are very difficult to obtain, forcing structural biologists to employ heterogeneous samples of GAG ligands. This often prevents crystallization of the protein-GAG complex. Solution NMR has been widely used in studying protein-GAG interactions, but the numerous dynamic interactions between protein and GAGs, as well as their electrostatic nature, limits the amount of direct data for these interactions. One of the problems in the study of protein-GAG interactions is direct confirmation of the GAG-binding sites on GAG-binding proteins. Although chemical shift perturbation analysis has been widely used to infer
ligand-binding in NMR, they, in fact, only reflect changes in magnetic environment. The work in this thesis was made possible by the use of paramagnetically-tagged GAG ligands. However, they require additional functionalization of the ligand. In this chapter, we explore the use of surface perturbation by an acidic paramagnetic agent as a possible method for determining GAG-binding sites on proteins.

Our method relies on the fact that protein-GAG interactions are mostly electrostatic in nature with basic clusters on protein surfaces often being the most likely GAG-binding sites. Therefore, a stable, negatively-charged nitrooxide radical may be used to probe the protein’s surface to identify the basic motifs involved in forming the protein-GAG complex [53, 77]. The magnetic moment of the paramagnetic molecule produces a strong local magnetic field that influences relaxation rate of the nucleus. This enhancement of the relaxation rate is useful for two reasons: first, it is distance-dependent, but unlike NOE, it can give long-range distance information up to 20 Å; second, it is directly proportional to the square of the gyromagnetic ratio, making $^1$H a sensitive probe [77]. In the absence of GAGs, these ligands will bind to the basic clusters on the protein surface, subjecting residues in the site to PRE. With the addition of GAG ligands, the radical will be excluded from sites on the protein surface, eliminating the PRE effect (Figure 5.1).
This ‘protection’ from PRE offered by GAGs can, therefore, be used as an indicator of GAG-binding, and residues protected by GAGs can be reasonably inferred as being close to the GAG-binding site. In the present study, the negatively-charged species, 4-carboxy-TEMPO, will be tested as a possible probing agent for identifying GAG-binding sites in proteins [77, 78].

The present study uses CCL5 (RANTES), a chemokine that controls the migration and activation of leukocytes [76], and the C-terminal domain of pleiotrophin (PTN-C), a cytokine whose full-length counterpart is responsible for early development of the nervous system, mitogenesis and angiogenesis [79], as the GAG-binding proteins of interest. CCL5’s interaction with GAGs, as well as its structure, has been well studied and it binds GAGs with high affinity ($K_D \sim 0.1 \mu M$ for CS hexasaccharides) [76]. CCL5 binds heparin, DS and CS through basic residues in the 40s loop (R44, K45 and R47) as well as residues around R17 in the N-loop and in the N-terminus [74, 76]. In comparison,
PTN-C binds heparin hexasaccharides with a $K_D$ of ~10 µM. This study shows that the residues involved in GAG-binding for CCL5 were protected from carboxy-TEMPO by CS tetrasaccharides, indicating that carboxy-TEMPO may be a good probe for GAG-binding epitopes. Preliminary data for PTN-C shows a similar trend to that of CCL5 with the identification of specific residues that experience protection.

5.2 Experimental Procedures

5.2.1 Expression and Purification of E66S CCL5 and PTN-C

The protocol for obtaining E66S CCL5 (a dimeric CCL5) was conducted in a similar manner to that of Czaplewski et al. [76, 80]. The ORF of mature CCL5 with the E66S mutation was cloned into pET23a then transformed into *E. coli* BL21(DE3) pLysS, resulting in a protein that, in addition to the E66S mutation, also contained an N-terminal methionine which differs from the mature form of CCL5. Then, the bacteria were grown in M9 minimal media at 37°C to an OD$_{600}$ of 0.5. The M9 was supplemented with $^{15}$NH$_4$Cl and glucose to provide isotopically-labeled protein. The bacteria were induced with 0.5 mM IPTG overnight at 28°C, then were harvested and lysed with sonication. The expressed protein was found in inclusion bodies, so to solubilize and refold the protein, the inclusion bodies were incubated with 0.1 M Tris (pH 8.0), 6 M guanidine hydrochloride (GnHCl). To remove additional large molecular weight contaminants from the solubilized protein, the inclusion body material was purified using a Superdex 200 size exclusion chromatography column (GE Life Sciences). The fractions containing the medium molecular weight E66S CCL5 were combined and refolded through a fast dilution protocol in which the protein was added dropwise to a refolding buffer (0.1 M Tris pH 8.0, 100 µM reduced glutathione, and 10 µM oxidized glutathione) that had a
volume ratio (buffer:CCL5) greater than ten to ensure proper refolding. The mixture was stirred overnight at 4°C and was then dialyzed against 20 mM acetic acid for 24 hours. The dialyzed protein was filtered and then injected onto a 5 mL SP strong cation exchange column (GE Life Sciences) equilibrated with 20 mM acetate (pH 4.0). The protein was eluted with a salt gradient from 0 M to 1.5 M NaCl and then dialyzed against 50 mM acetate (pH 4.4) buffer.

The ORF of PTN-C (residues 90-146) was cloned into the pHUE vector, resulting in a fusion protein with N-terminal His-tagged ubiquitin [42], and then each plasmid was transformed into E. coli BL21(DE3). To isotopically label the protein, the bacteria were grown at 37°C in M9 minimal media supplemented with $^{15}$NH$_4$Cl and glucose until an OD$_{600}$ of 0.5 was reached. The bacteria were induced with 0.5 mM IPTC then incubated overnight at 30°C. After harvesting, the cells were lysed with a combination of incubation with 1 mg/mL lysozyme and sonication. The fusion protein found in the cleared supernatant was extracted using Ni affinity chromatography with a 5 mL HisTrap column (GE Life Sciences) and an imidazole gradient of 25 mM to 300 mM at a flow rate of 3 mL/min. The fractions containing the fusion protein were combined and exchanged into 25 mM Tris (pH 8.0), 100 mM NaCl. Then, the fusion protein was digested overnight at room temperature with USP2 and 0.5 mM DTT. The cleaved PTN-C was eluted in the flow-through of a 5 mL HisTrap column and then was collected, concentrated and exchanged into 10 mM MES (pH 6.0), 150 mM NaCl. (Production and expression of PTN-C was conducted by Nate Kuch.)
5.2.2 Production of CS and Heparin Fragments

Heparin from porcine intestinal mucosa and CS from porcine trachea (Sigma Aldrich) were digested with 0.5 IU of heparinase I (IBEX Inc.) and 3 mg/mL type V hyaluronidase from sheep testes, respectively, to produce short fragments. The heparin digest was monitored at an absorbance of 232 nm until depolymerization was 30% complete while CS was digested for two days at 37°C. The short GAG fragments were separated with a 2.5 cm × 175 cm size exclusion chromatography column (Bio-Rad Biogel P10) at a flow rate of 0.2 mL/min. Fractions containing fragments of the same size were combined then desalted and lyophilized.

5.2.3 Acquisition and Analysis of PRE

NMR data for PRE experiments were collected on a Bruker Ultra-Shield 600 MHz spectrometer equipped with a triple resonance cryoprobe. To detect the surface exposed residues most likely to interact with GAGs, 2 mM 4-carboxy-TEMPO was added to 400 µL of 200 µM 15N-labeled E66S CCL5, while 10 mM 4-carboxy-TEMPO was added to 180 µL of 100 µM 15N-labeled PTN-C, and 1H—15N HSQCs were collected before and after reduction with 10 mM (E66S CCL5) or 20 mM (PTN-C) ascorbic acid. Additionally, 1H—15N HSQCs were collected for each protein sample containing two molar equivalents of GAGs (CS dp4 for E66S CCL5, hep dp6 for PTN-C) along with oxidized 4-carboxy-TEMPO and for each protein-GAG sample after the TEMPO had been reduced. The paramagnetic transverse relaxation rates (R_{2,PRE}) for each HSQC were estimated using Equation 1.8, then the paramagnetic contribution to transverse relaxation was measured as the difference between the R_{2,PRE} values before and after reduction of
the TEMPO radical in the presence and absence of GAGs. (Acquisition of PTN-C HSQC spectra was done by Nate Kuch.)

5.3 Results

A comparison of the $^1$H—$^{15}$N HSQC spectra of E66S CCL5 with oxidized and reduced carboxy-TEMPO (Figure 5.2) shows several residues are strongly affected by the addition of the nitroxide radical (R17, K46 and R47). Crosspeaks for these residues completely disappeared in the spectrum when ten molar equivalents of carboxy-TEMPO was added to E66S CCL5. It should be noted that these same residues are also involved in GAG-binding.

![Figure 5.2. Comparison of HSQC spectra of E66S CCL5 with reduced carboxy-TEMPO (left), with oxidized carboxy-TEMPO (middle) and with oxidized carboxy-TEMPO and CS dp4 (right). Crosspeaks are labeled with the corresponding assignments.](image)

The crosspeaks of other residues in the same spectrum were affected to a much smaller extent, indicating that they are farther away from the radical. Specifically, K55 and R59 experienced minimum perturbation despite the fact that they are basic residues, which should be the prime target for GAGs and other anionic species. Upon the addition of CS dp4, the crosspeaks for R17, K46 and R47 reappeared due to the displacement of carboxy-TEMPO by CS. The $R_{2,PRE}$ produced by reduced carboxy-TEMPO with and
without CS dp4 were estimated from HSQC peak intensities and line widths according to Section 1.4.5 and Equation 1.8. The difference between the $R_{2,\text{PRE}}$ values with and without CS dp4 give the CS dp4-induced decrease in $R_{2,\text{PRE}}$, which is due to the protection by CS dp4 from the paramagnetic center, and is referred to as $R_{\text{protect}}$. The effect of this protection is shown in Figure 5.3.

Figure 5.3. Surface protection PRE on backbone amide protons of E66S CCL5 of CS dp4 from 4-carboxy-TEMPO. Residues in the known binding site experienced the most protection as compared to the non-GAG-binding regions of the protein. $R_{\text{protect}}$ values of the residues are mapped onto the ribbon diagram of E66S CCL5 with a white-to-red gradient that reflects $R_{\text{protect}}$ values of 10 to 70 s$^{-1}$.

For most of the residues in E66S CCL5, the amide proton experienced small $R_{\text{protect}}$ between -5 and 20 s$^{-1}$. However, the residues in the known GAG-binding site (R17 and L19 in the N-loop, Y3 in the N-terminus and R44, K45 and R47 in the 40s loop) experienced severe, but measurable, protection effects.

Preliminary data for PTN-C shows that binding GAGs also results in protection from effects induced by 4-carboxy-TEMPO. The effect of heparin dp6 on $^1$H—$^{15}$N HSQC spectra of PTN-C in the presence 4-carboxy-TEMPO can be seen in Figure 5.4.
PTN-C experienced three areas of severe PRE upon addition of carboxy-TEMPO: the first is G96, C99 and F103; the second is K123 and R124; and the third is G142. Like the E66S CCL5 data, most of the residues did not experience PRE, indicating that the residues that do experience severe PRE are most likely part of a basic cluster that can interact with carboxy-TEMPO. Based on other studies of PTN-GAG interaction, the residues noted above also play a role in heparin-binding to PTN-C but further
experimentation is required to validate this as well as the GAG-binding site for the intact PTN protein.

5.4 Discussion

There is very little structural information for protein-GAG complexes due to the dynamic nature of the interaction and the heterogeneity of the GAGs themselves. GAGs interact with a protein through electrostatic interactions with basic amino acids but the amount of these residues is not necessarily a good predictor of GAG-binding motifs [5]. Probing the protein surface with the nitroxide radical then observing the protection afforded to the protein through GAG-binding allows for early identification of potential GAG-binding sites. Therefore, this method could be used in conjunction with structural determination to quickly identify residues for mutagenesis studies required for further biochemical experiments. This study used E66S CCL5, which has a well-studied GAG-binding site, to test the efficacy of this method. The residues experiencing the largest protection were located in the 40s loop, the N-loop and the N-terminus of E66S CCL5 which form the GAG-binding site [74, 76]. This result indicates such a method can accurately and unambiguously determine the GAG-binding site in the protein.

Interestingly, not all basic amino acids in E66S CCL5 interacted with carboxy-TEMPO as strongly as the GAG-binding residues. In particular, K55 and R59 are hardly perturbed by the presence of this acidic paramagnetic ligand. This selectivity by carboxy-TEMPO mirrors that of GAGs precisely. Examination of the CCL5 structure offered a possible explanation of this phenomenon: unlike the GAG-binding basic residues, K55 and R59 are adjacent to acidic residues that are in position to ion-pair with them. This observation shows that, at least in the case of E66S CCL5, availability of basic residues to
electrostatically interact with GAGs is a strong determinant of GAG affinity. However, other factors may also be in play. In particular, residues in the N-terminus and N-loop are also implicated in binding both GAGs and carboxy-TEMPO, but these segments do not have as many basic residues as the 40s loop. Despite this, they are enriched with polar amino acids capable of hydrogen-bonding with GAGs. This could be another factor in their affinity for both GAGs and carboxy-TEMPO.

The preliminary investigation into the GAG-binding domain of PTN-C revealed specific areas that experienced protection when PTN-C interacted with heparin dp6. We performed a study on PTN-C’s interactions with GAGs that, similar to the case of E66S CCL5, showed these residues are also part of a GAG-binding site. This confirms the utility of carboxy-TEMPO as a general tool for determining GAG-binding sites in proteins. However, more quantitative experiments will be needed to confirm the observation. It should also be noted that similar experiments carried out on intact PTN produce much more ambiguous results (data not shown). We believe that this is a result of at least one factor. Specifically, PTN has two domains capable of binding GAGs with similar affinities. The competition for GAG ligands between them will, undoubtedly, make the interaction between PTN-C and GAGs more dynamic. Indeed, the apparent $K_D$ of interaction for PTN-C in intact PTN is $\sim$60 $\mu$M, far higher than the value of $\sim$10 $\mu$M measured for PTN-C on its own. The possibility that the presence of the other domain may obstruct access to PTN-C’s GAG-binding site also cannot be ruled out. This apparent decrease in GAG-affinity for PTN-C most likely made the protection effect less detectable.
CHAPTER 6
Conclusion and Future Work

Work in this thesis has achieved a thorough understanding of the GAG binding mechanisms of DBPA and has contributed to the rationalization of the dependence of Lyme disease symptoms on DBPA sequence heterogeneity. However, several aspects of DBPA structure and function are still to be explored. In particular, the functional differences between soluble and lipidated forms of the protein have not been investigated, and the role of decorin core protein in promoting DBPA adhesion is not well understood.

6.1 Effect of DBPA Lipidation on GAG-Binding

The experiments in this thesis were conducted with the soluble, mature form of DBPA which lacks the N-terminal triacyl-modified cysteine [81, 82]. This lipidation is what allows DBPA to be anchored to the outer membrane of the *Borrelia* spirochete. The lipidated version of DBPA could have different oligomerization states or even tertiary structure than the soluble, non-lipidated form. A study done by Ulbrandt et al. showed that the lipidated form of N40 DBPA bound to immobilized, intact decorin with substantially higher affinity than did the soluble, His-tagged DBPA at the same concentrations [83]. Preliminary studies carried out by us shows this difference in binding affinity may be partially due to the oligomerization of free lipidated DBPA. In particular, $^{15}$N—HSQC of the protein shows lipidated DBPA exists as large oligomers in the absence of detergents. When incorporated into a membrane mimic like a DHPC micelle, this large oligomer is presumably solubilized and DBPA’s affinity for decorin decreases but still remains higher than that of soluble DBPA (data not shown). A comparison of $^{15}$N—HSQC spectra from lipidated DBPA with DHPC micelle and soluble
DBPA verified that the micelle disrupts protein aggregation, giving a spectrum that is similar to the one seen with soluble DBPA. Therefore, the lipidation may not have a large effect on the tertiary structure of DBPA, but it does increase binding affinity of DBPA for intact decorin through oligomerization. In addition to the GAG-binding epitopes found in the various strains of DBPA, immobilization of DBPA on the surface of the spirochete also plays a role in GAG-binding affinity of the protein. In particular, the oligomerization state of the protein on the natural membrane of the spirochete bacterium is not known. Further study into this effect could lead to further understanding of the structural mechanisms through which DBPA binds GAGs.

6.2 Interaction of DBPA with Decorin

While DBPA has been shown to interact with the DS chains found on decorin, several studies have proven that DBPA binds intact decorin much tighter than DS [14-16, 19, 26]. Recently, Lin and co-workers showed with ELISA that B31, N40 and PBr bound to decorin with a $K_D$ of 0.21 µM, 0.85 µM and 0.06 µM, respectively; whereas, the same three strains of DBPA bound to intact DS with a $K_D$ of 0.91 µM, 3.10 µM and 0.21 µM, respectively [26]. Initially, the difference in this affinity was attributed to a potential interaction of DBPA with the core protein of decorin [14, 19]. ELISA conducted by us shows the decorin core protein alone is unable to bind DBPA at all (data not shown), indicating that DBPA’s interaction with decorin is primarily through the GAG chains.

A possible explanation for the difference in DBPA’s affinity for intact decorin and DS is that, in vivo, decorin exists as a dimer which means there are two DS chains available for DBPA binding [50, 84]. The presence of an additional DS chain could induce additional DBPA oligomerization and increase binding affinity for intact decorin.
Another possible explanation is that, although there is interaction between the decorin core protein and DBPA, it is too weak to be measured by ELISA but can contribute to the enhancement of the DBPA-decorin interaction through the avidity effect as part of the proteoglycan. It is also possible that the quaternary structure of decorin presents GAG in such a way as to dramatically increase the affinity of the GAGs for DBPA. An additional possibility is that the difference in affinity seen in these ELISA is an artifact of differences in plating efficiency of decorin and DS. If decorin can be immobilized with much higher efficiency than DS, and in our experience this is indeed the case, then the assumption of insignificant depletion of the soluble DBPA used to extract the $K_D$ value may not be valid in the case of decorin. At the moment, high resolution investigation of DBPA-decorin interaction is not yet feasible since obtaining decorin with homogeneous glycosylation is not possible, but the knowledge of how the core protein of proteoglycans can influence GAG-protein interactions will be invaluable.

6.3 Optimization of Probing Protein-Glycosaminoglycan Binding Sites

Experiments similar to those done in Chapter 5 were attempted on B31 DBPA, a weaker GAG-binding protein compared to E66S CCL5 and PTN-C, but little protection effect was observed (data not shown). This indicates that the robustness of the method relies on two factors: strong interactions between 4-carboxy-TEMPO and the protein as well as strong interactions between GAG ligands and the protein. E66S CCL5 and PTN-C both satisfy the above criteria. In comparison, the lower GAG affinity of B31 DBPA, with its accompanying low affinity for carboxy-TEMPO, means the method under current conditions is not sufficient to reveal the GAG-binding site. However, use of another,
stronger paramagnetic agent, like Gd-DTPA, and optimization of the experimental conditions may make the method applicable to all GAG-binding proteins.
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