

Binding of a de novo designed peptide to specific glycosaminoglycans

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Abstract The binding of glycosaminoglycans to a synthetic peptide (SKAQKAQAKQAKQAQKAQAKQAKQW-CONH₂), consisting of a hybrid consensus heparin binding sequence, is studied using circular dichroism, fluorescence anisotropy and nuclear magnetic resonance techniques. The results unveil certain novel features, most importantly, the peptide binds preferentially to iduronic acid containing glycosaminoglycans and the dissociation constant for the peptide–heparin complex was found to be 30 nM. Interestingly, higher order intermolecular association(s)/aggregation was not observed, especially at saturating concentrations of the ligand. The helical structure of the peptide backbone, induced upon binding to a particular glycosaminoglycan is directly related to their binding affinity. In our opinion, studies on such unconventional hybrid peptide sequences containing low density basic amino acid residues would lead to the design of sequence specific glycosaminoglycan binding peptides. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: De novo design; Lysine rich peptide; Glycosaminoglycan; Induced helix; Binding specificity; Electrostatic interaction

1. Introduction

Glycosaminoglycans have been demonstrated to mediate a wide range of biological activities such as cell adhesion, cell motility, cell proliferation and tissue morphogenesis by binding to various cell regulatory proteins such as the chemokines, growth factors, enzymes, enzyme inhibitors, extracellular matrix proteins, toxins and proteins from microbial agents [1–8]. It is well accepted that the electrostatic interaction between the sulfates of the glycosaminoglycan chain and the basic residues on the protein contribute significantly to this biomolecular recognition process [2,8]. Studies have been carried out using model peptides containing either lysine/arginine rich or those abiding the consensus heparin binding amino acid sequence(s) (XBBXB or XBBBXXB). The results of these studies, in conjunction with the statistical analysis of the heparin binding amino acid sequences in proteins, indicate that the spatial distance between the basic amino acid residues is the major determinant involved in the binding process [8–13]. In spite of the growing importance of the protein–glycosaminoglycan recognition process none of the studies [8,11,13,14], until date, have addressed the differential binding ability of the designed peptides to various glycosaminoglycans. It is possible that the high density of the positively charged resi-

dues on the designed peptides would, predictably, hinder the selectivity in binding. Therefore optimization of the number and position of the positively charged amino acid residues in the designed peptides is essential to achieve substrate specificity. In this context, we embarked upon studying the interaction of various glycosaminoglycans with a low density lysine peptide (K8) using circular dichroism, fluorescence anisotropy and nuclear magnetic resonance techniques. We demonstrate that the designed peptide binds preferentially to iduronic acid containing glycosaminoglycans with reasonably high affinity.

2. Materials and methods

The de novo designed 28-residue low density lysine peptide (K8: SKAQKAQAKQAKQAQKAQAKQAKQW-CONH₂) was synthesized at SynPep Corp. (CA, USA) and was further purified using a semipreparative C₁₈ reverse phase HPLC column with appropriate water–acetonitrile gradient containing 0.1% TFA. The authenticity of the peptide was confirmed by amino acid analysis and mass spectrometry. Low molecular weight heparin (Hep), heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) and hyaluronic acid (HA) were purchased from Sigma (USA). Titrations of K8 with different GAGs were performed in 10 mM phosphate buffer (pH 7.4) at 25°C, unless otherwise mentioned. The titrations were carried out by adding aliquots of a particular GAG to a constant concentration of K8. The concentration of the peptide was determined from its extinction coefficient of 1280 M⁻¹ cm⁻¹ at 280 nm [15]. The concentration of Hep was determined by the uronic acid assay [16].

2.1. Fluorescence measurements

Anisotropy measurements were made using an SLM 4800 fluorimeter with an excitation and emission wavelength of 280 nm and 350 nm, respectively. The fluorescence intensity (*I*) and the gain (*G*) were obtained in parallel and perpendicular modes. The fluorescence anisotropy values were calculated using the relation $R = (R_V - R_H) / (R_V + R_H)$ where $R_H = G_{\parallel}I_{\parallel} / G_{\perp}I_{\perp}$ and $R_V = G_{\parallel}I_{\parallel} / G_{\perp}I_{\perp}$ [17,18].

2.2. Circular dichroism spectroscopy

All CD measurements, in the far UV region (195 nm to 260 nm) of the spectrum, were made using a 1 cm pathlength cell on an AVIV CD spectropolarimeter equipped with a programmable heating unit. The spectropolarimeter was calibrated using d₁₀-camphor sulfonic acid. The thermal stability of the peptide upon binding to various glycosaminoglycans was inferred from the changes in the mean residue ellipticity values at 222 nm. In order to evaluate the van't Hoff enthalpy associated with the complex formation, the titration of the peptide (K8) with heparin was performed at different temperatures (288 K, 293 K, 298 K, 303 K and 308 K).

2.3. Data analysis

The apparent dissociation constant values were estimated from the changes in the fluorescence anisotropy upon titrating K8 with a given glycosaminoglycan, using the non-linear relation

$$F = F_o + [(F_s - F_o) / 2P_t] [(K_d + nL_t + P_t) +$$

$$((K_d + nL_t + P_t)^2 - 4nL_tP_t)^{0.5}]$$

where *F*_o and *F*_s represent the anisotropy values of the peptide in the absence and presence of saturating concentrations of the ligand (gly-

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cosaminoglycan); P_t and L_t are the total concentrations of the peptide and the ligand, respectively; K_d is the apparent dissociation constant of the peptide–oligosaccharide complex.

2.4. NMR spectroscopy

One-dimensional ^1H -NMR of K8 (1 mM in 10 mM sodium phosphate buffer, pH 7.4), at different concentration of low molecular weight heparin, was measured on a 500 MHz NMR spectrometer using a spectral width of 6000 Hz. Each spectrum was an average of 64 scans and was referenced to 3-(trimethylsilyl)propionate. ^{15}N -HSQC [19] experiments were performed on a ^{15}N -Ala-K8 (all alanines labeled with ^{15}N) with a spectral width of 100 ppm (512 data points) and 4 ppm (2048 data points) in F2 and F1 dimensions, respectively. The spectra were processed using XWIN-NMR software.

3. Results and discussion

3.1. Peptide design

The heparin binding domains of a few proteins, notably thrombospondin [20], antithrombin [21], vitronectin [22], IGFBP-5 [23] and IGFBP-3 [24], contain the basic amino acid residues arranged in an $(i,i+3)$ order. This enables the basic residues to orient in nearly the same direction, provided the secondary structure is α -helix and are thus conducive for binding to negatively charged ligands such as glycosaminoglycans. Studies on synthetic peptides derived from the heparin binding domain(s) of these proteins indicate a change in the backbone conformation of the peptide upon binding to heparin. Complementarily, the oligosaccharide derived from heparin has been shown to adopt a helix-like conformation [25,26]. With these as a clue, we designed a peptide with lysines at alternate positions of three and four. Such an arrangement of the basic residues represents a hybrid of the heparin binding consensus sequences proposed by Cardin and Weintraub (XBBXB and XBBBXXB). Helical wheel representation of the amino acid sequence (not shown) of K8 indicates that the lysines are arranged on one side of the helical axis. Therefore, it is envisaged that the peptide could adopt α -helical structure upon binding to glycosaminoglycans. Glutamine and alanine were opted as the intervening residues as they have high intrinsic propensity to adopt helical conformation, under favorable conditions [27–30]. The inclusion of trypto-

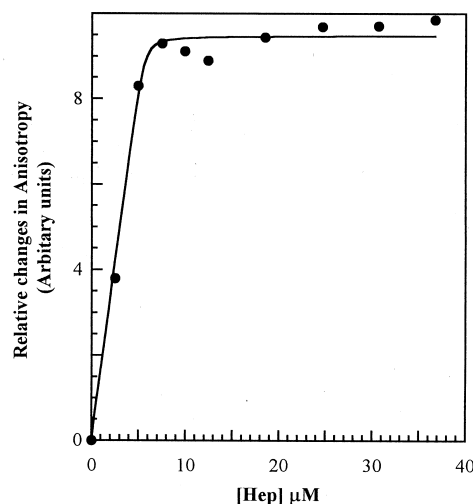


Fig. 2. Changes in the anisotropy values of K8 upon titrating with Hep. The best fit was obtained with $K_d = 30$ nM and $n = 1$ in the non-linear equation described in Section 2.

phan served dual purpose, as a probe for the estimation of peptide concentration and also for fluorescence anisotropy measurements.

3.2. Titration with heparin

The binding strength of K8 was initially examined using immobilized heparin (HiTrap, Pharmacia). It was observed that 0.7 M of NaCl was required to elute K8 from the heparin affinity column. In general, proteins/peptides that could be eluted with more than 1.0 M NaCl are considered to possess high affinity for heparin and those required less than 0.3 M NaCl are predicted to exhibit weaker binding. This indicates that the designed peptide, K8, binds quite significantly with heparin. The peptide was also titrated with soluble heparin and its binding was monitored by observing the changes in the far UV region of the circular dichroic (CD) spectra and also by the changes in the fluorescence anisotropy values. The CD spectra (in the far UV region) exhibited absorption bands at 195 nm and 214 nm implying that the intrinsic backbone conformation of K8 was an extended charged coil (Fig. 1). This appeared to be a characteristic feature of highly charged peptides [31,32]. Upon heparin addition, double minima at 206 nm and 222 nm with concurrent appearance of a maximum at 195 nm seems to develop in the CD spectra of the peptide (Fig. 1). This indicates that the designed peptide, K8, does bind to soluble heparin and that the backbone adopts an α -helical conformation upon binding [33]. Such conformational changes have been reported for several heparin binding segments of proteins and also for synthetic peptides upon binding to heparin [8,14,31]. In addition, we monitored the binding of K8 with Hep from the changes in the fluorescence anisotropy of the peptide. The dissociation constant, calculated from this isotherm (see Section 2), was found to be 30 nM (Fig. 2).

In order to authenticate the binding of K8 to Hep and also to examine the possibility of the formation of higher order molecular associates, the interaction of K8 with Hep was also followed by monitoring the changes in the proton resonances of the peptide during the titration. The amide proton resonances of the peptide exhibited a gradual dispersion with si-

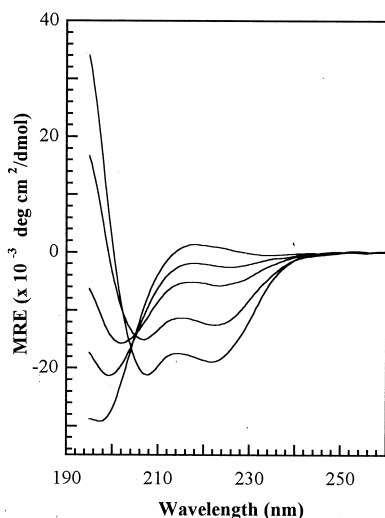


Fig. 1. Far UV region of the CD spectrum of 8K34 (5 μM) at different concentrations of Hep. Heparin concentration from top to bottom (at 222 nm) is 0 μM , 1 μM , 2 μM , 4 μM and 10 μM .

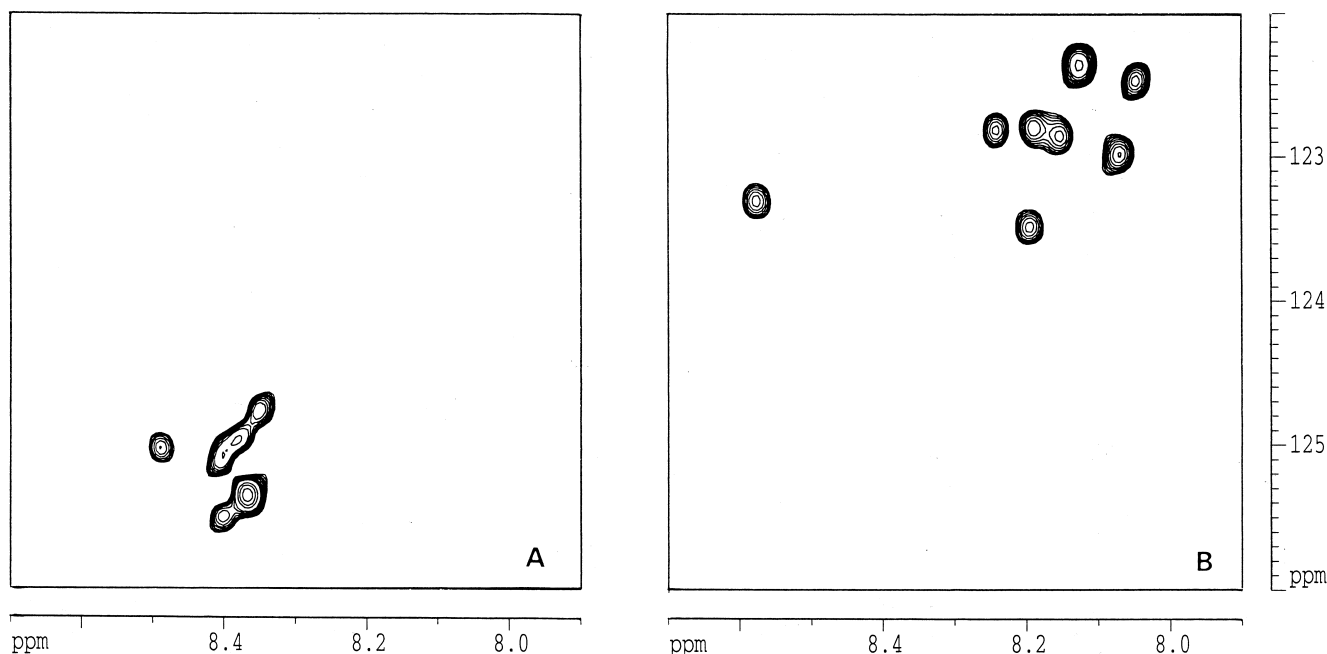


Fig. 3. ^{15}N -HSQC spectra of ^{15}Ala -8K34 in the absence (A) and presence (B) of Hep. The transition from the unstructured to an ordered conformation for 8K34 upon binding to Hep is evident from the increase in the dispersion of the cross-peaks both in ^1H and ^{15}N dimensions.

multaneous upfield shift. This clearly indicates that the peptide backbone adopts an α -helical structure upon binding to heparin [34,35]. As the amide protons are not well resolved in the 1D spectra, we acquired the ^{15}N -HSQC spectrum of the peptide (^{15}N -Ala-K8) in the absence and presence of saturating concentrations of heparin (Fig. 3). The dispersion of the proton resonances is more pronounced in the ^{15}N -HSQC spectrum. Moreover, the line widths of these cross-peaks, both in the absence and presence of heparin, did not exhibit dramatic changes. These results clearly emphasize the absence of higher order intermolecular associates, especially at saturating concentrations of heparin.

3.3. Titration with other glycosaminoglycans

The peptide was titrated with various glycosaminoglycans (HS, DS, CSA, CSC and HA). The CD spectrum of the peptide does exhibit changes (formation of double minima at 206 nm and 222 nm) in the far UV region when the titrant was either HS or DS (Fig. 4). Interestingly, no significant changes in the CD signals were observed when the peptide was titrated with CSA (Fig. 4). It should be mentioned that the marginal changes in the ellipticity values of K8, observed upon titrating with either CSC or HA are not associated with the formation of helix. This small increase could be due to the presence of higher molecular associates owing to the possible formation of very weak/non-specific peptide-CSC/HA complexes. Therefore, the results obtained from the circular dichroism spectra indicate that the peptide interacts with HS and DS (in addition to Hep) but not with CSA, CSC and HA. To authenticate this result, fluorescence anisotropy measurements were made upon titrating the peptide with glycosaminoglycans. Changes in the anisotropy values for K8 were observed upon titration with DS and HS but not with CSA (data not shown). The changes in the anisotropy values associated with the titration of K8 with either CSC or HA could be due to non-specificities in the interaction. Therefore the results of the

fluorescence anisotropy measurements corroborate well with the data obtained from the circular dichroism measurements, thus demonstrating that the peptide preferentially interacts with HS and DS. Analysis of the molecular structure of these different glycosaminoglycans reveals that Hep, HS and DS contain iduronic acid as the hexuronic acid moiety whereas the other glycosaminoglycans used in this study have the corresponding epimer, glucuronic acid. It has to be emphasized that Hep and HS contain a mixture of glucuronic acid and iduronic acid sugars. Lack of binding by HA could be attributed to the lack of sulfation of the hydroxyls, in addition to the presence of glucuronic acid. Such a differential binding ability might also be due to higher conformational flexibility

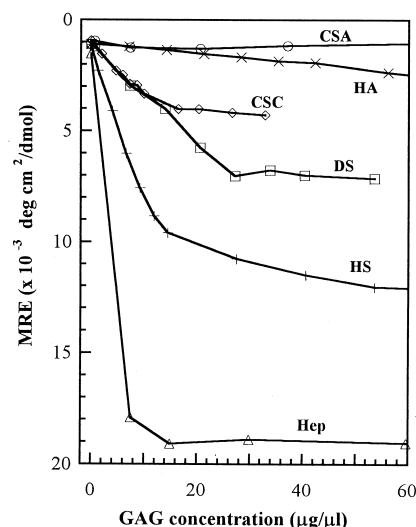


Fig. 4. Changes in the mean residue ellipticity values of 8K34 (at 222 nm) upon titrating with different GAGs. The titrations were performed in 10 mM phosphate buffer, pH 7.4.

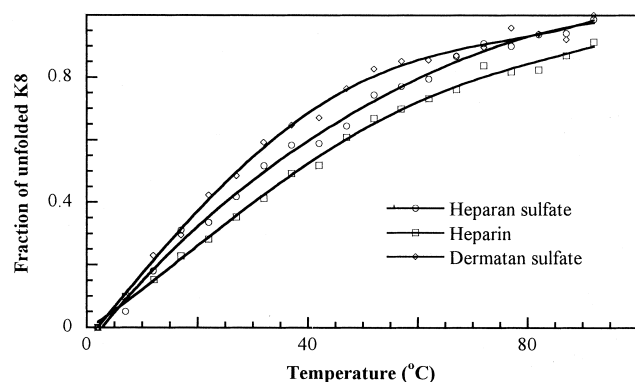


Fig. 5. Changes in the fraction of unfolded species of 8K34 upon increasing the temperature. The fraction of unfolded species was calculated based on the mean residue ellipticity values at 222 nm. The denaturation was monitored under saturating concentrations of Hep, HS and DS.

of the iduronic acids than compared to a seemingly rigid conformation of glucuronic acids [36]. Thus, it could also be envisaged that an induced fit mechanism used to explain the peptide–glycosaminoglycan interaction requires not only the changes in the peptide backbone conformation but also the sugar chain. Therefore, the results of CD and fluorescence anisotropy confine to the view point that the designed peptide, K8, interacts with those glycosaminoglycans that lodge the iduronic acid sugar.

In order to assess the relative binding strengths of the peptide with glycosaminoglycans, the thermal melting curves of the peptide, in the presence of saturating concentrations of the iduronic acid containing glycosaminoglycans, were analyzed. The thermal melting curves did not exhibit a sigmoidal trace, which is quite expected of peptides lacking rigid tertiary structures (Fig. 5). However, qualitative analysis indicates that the stability of the complexes decreases in the order $\text{Hep} > \text{HS} > \text{DS}$ (Fig. 5). The trend could also be correlated to the inherent differences in the sulfation pattern of these glycosaminoglycans. As DS is the least sulfated among the iduronic acid containing glycosaminoglycans used in the present study, the binding appears to be relatively weaker. It should also be stated that a clear correlation between the percentage of the helix induced to the corresponding binding strength is observed. Such trends have also been observed with the heparin binding studies using Cardin–Weintraub sequences [31].

3.4. Comparison with other studies

The peptide used in the present study has been demonstrated to preferentially bind to iduronic acid containing glycosaminoglycans. This is in contrast to the fact that the homopolymers of lysine/arginine/ornithine could bind to all glycosaminoglycans [32]. This could imply that the high conformational flexibility of the iduronic acid sugars cannot be used exclusively for explaining the substrate specificity. The disparity in the binding preferences observed by Gelman and Blackwell and in the present study could be attributed to the nature of the peptides used for the study. Irrespective of the glycosaminoglycan used, the homopolymers adopted helical conformation upon complex formation. In contrast, we find that the helical conformation is induced in K8 only upon interacting with the iduronic acid containing glycosaminogly-

cans. Therefore these studies indicate that helix induction accompanies the binding reaction. It should be noted that there is no preferential distribution of the charges in the case of homopolypeptides, owing to which the non-specificities in the interaction are higher. On the other hand, the structure of K8 is more ordered with the positive charges distributed on one side of the helix. Such an orientation of the charges would definitely minimize the non-specificities in the interaction with glycosaminoglycans. Moreover, the studies with polylysine and polyarginine clearly indicate the presence of higher molecular associates or aggregates [32]. Formation of aggregates (or higher molecular associates) is a major concern in the study of peptide/protein glycosaminoglycan studies. This phenomenon could arise due to mere charge neutralization, upon binding, which would lead to poor solubility of the complex or due to the occurrence of non-specific interactions. It is quite interesting to note that the results of the present study vividly demonstrate the absence of higher molecular associates.

Heparin binding studies using the peptides, consisting of the tandem repeats of Cardin–Weintraub motifs have been carried out recently [31]. The results of this study imply that the binding behavior of the peptide to heparin is largely dependent on the peptide:heparin ratios. The authors observe that the helical conformation of the peptide that is induced at lower concentration of heparin, melts when the molar ratio of the heparin/peptide is greater than one. On the contrary, the present study on K8 indicates that the K8–heparin complex is quite stable and the induced helical conformation persists even in the presence of excess molar concentrations of heparin. Such differences in the binding behavior could be attributed to either (a) the spacings between the positively charged lysine residues or (b) the intervening amino acid residues. Verrechio et al. [31], based on the studies using different mutant peptides, claim that the intervening residues (except the presence of proline/glycine) do not have prominent role in the binding process. In the present study, we deduced the change in the van't Hoff enthalpy associated with the interaction of K8 with heparin. A value of -2.94 kcal/mol (ΔH) associated with the formation of K8–heparin complex is comparable to those obtained in the heparin binding studies using short peptides [11,14]. Such lower values for ΔH imply that the electrostatic forces dominate in the interaction of K8 with heparin and that the presence of either glutamine or alanine does not contribute to the binding of K8 with heparin. Therefore, it is obvious that the patterns and spacings of the lysine residues in the peptides used in these studies ([31] and present study) play a significant role in dictating the binding behavior. Therefore, in our opinion, the density of the positively charged residues, in addition to the interresidual spacings could be a very crucial factor in the peptide–heparin recognition process.

In conclusion, the present study paves way for the design of non-conventional heparin/glycosaminoglycan binding amino acid sequences. These sequences which do not contain dense clusters of basic residues ($< 30\%$ compared to $> 50\%$ in conventional heparin binding sequences) might enable specificity in the glycosaminoglycan recognition. Such specificities are duly important in the design of high affinity peptides to a given glycosaminoglycan sequence which could ultimately find application in modulating the biological processes in the cell, mediated by glycosaminoglycans.

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