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Heparin Dependent Coiled-Coil Formation

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Heparan sulfate (HS) is a ubiquitous glycosaminoglycan that has been demonstrated to mediate protein complex formation in the extracellular matrix essential to many signaling pathways.[1–3] HS is a linear polysaccharide with a highly heterogeneous structure caused by extensive enzymatic modifications carried out during its synthesis.[4] The modifications result in domains along the polymer backbone of high sulfation density (S domains) separated by tracks of polysaccharide with a low degree of sulfation (NA domains).[5] How the biosynthesis of specific heparan structures are controlled and which structures are required to form specific heparan–protein complexes remains a key question in glycobiology.[1] The highly sulfated S domains in HS closely resemble the common anticoagulant heparin in their structure and sulfation density. In this study heparin is used as a model of the highly sulfated S domains in HS.

Herein we report coiled-coil–heparin complexes that emulate the ability of HS to promote multimeric protein complex formation. The binding of heparan and other polyanions to positively charged peptides is a well-known phenomenon. It is these polyelectrolyte interactions that have been proposed to be the basis of cell-penetrating-peptide binding at the cell surface,[6] the antibacterial action of cationic peptides on anionic membranes,[7] and the formation of DNA delivery complexes.[8] The cationic peptide system we report herein uses heparin binding to simultaneously direct a peptide conformational change and mediate the formation of a dimeric coiled-coil complex. These designed coiled-coils may serve as models to study HS–protein complex formation and serve to demonstrate the potential of using heparin binding as an element in protein design.

Coiled-coils are the best studied protein tertiary structures which form stable folds, even with short peptides.[9–11] They have found extensive use in protein engineering and nanotechnology, as the factors important in the design of stable complexes are well understood.[12] Coiled-coils typically consist of two to five α-helices twisted into a left-handed helical supercoil. The supercoiling facilitates the formation of a densely packed hydrophobic core established by an amino acid heptad repeat (*abcdefgh*) containing hydrophobic residues at the *a* and *d* positions. Control over directionality, homo- versus heterooligomerization, and coiled-coil stability has been achieved by tuning the electrostatic interactions between the *e* and *g* residues flanking the hydrophobic interface.[13–26] We hypothesized that a linear anionic heparin polysaccharide would be of complementary size and shape to interact with positively charged amino acid residues displayed at the *e* and *g* positions of a designed coiled-coil (Figure 1).[27,28] A leucine zipper containing four lysine residues placed in the *b*, *c*, *f*, and *g* of positions of one heptad has previously been shown to have affinity for heparin.[29] In our peptides, charge repulsion between the *e* and *g* positions would destabilize the coiled-coil and prevent peptide dimerization in the absence of heparin. Cardin and Weintraub first proposed that α-helices bearing basic residues along one face could form a heparin binding site.[30] Subsequently it has been observed that the binding of peptides to heparin and other polyelectrolytes, can induce a conformational change toward an α-helix.[31–34] A similar conformational change in this system would promote coiled-coil formation by ordering the hydrophobic interface and by providing a strong favorable electrostatic interaction with heparin along the polycationic helices.

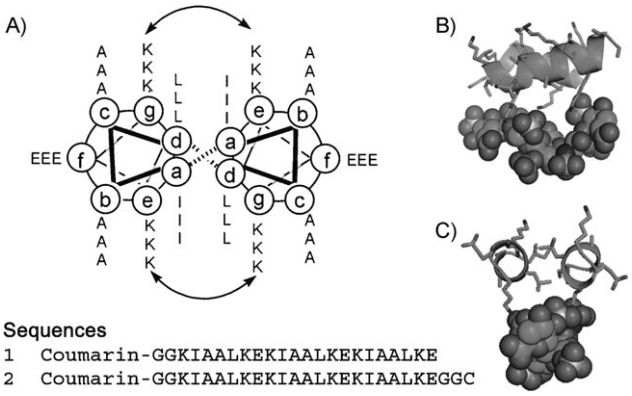

Figure 1. Heparin-dependent coiled-coil-forming peptides. A) Peptide sequence shown on a parallel coiled-coil helical wheel, arrows indicate electrostatic interactions. B) Pentasaccharide of heparin (space filling) and two heptad repeats of a parallel coiled-coil. C) End on view of B with only one heptad repeat shown. B and C are manually docked models.

To explore this possibility, peptide sequences were synthesized which have been shown by the Hodges group not to homodimerize because of electrostatic repulsion of the lysine residues at the *e* and *g* positions (Figure 1).[15] A 7-diethylaminocoumarin fluorophore was appended to the N terminus of these peptides to serve as a fluorescent probe of heparin binding and coiled-coil formation. This fluorophore has been attached to coiled-coils and has been shown to increase their association constant but does not change the oligomerization state of the complex.[35] A C-terminal disulfide bridged variant, peptide 2, was also synthesized to allow for further control over the stoichiometry of the complex.

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Complex formation between heparin and peptides 1 and 2 was evaluated by using CD spectroscopy, fluorescence titrations, and equilibrium ultracentrifugation in a physiologically relevant buffer (10 mM Tris, 100 mM NaCl, pH 7.0).

The CD spectrum of peptide 1 alone is indicative of a peptide with minimal helical structure, but upon the addition of heparin a strong increase in helicity is observed (Figure 2). The

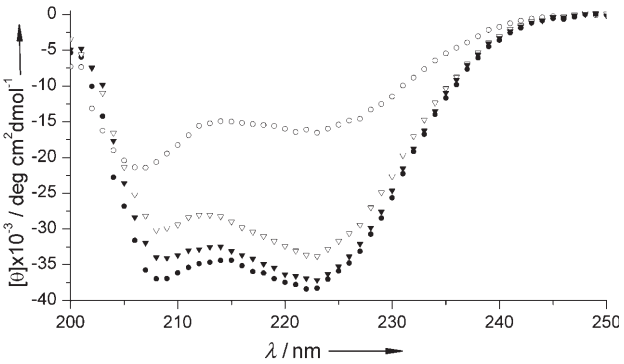

Figure 2. CD spectra of **1** (circles) and disulfide-linked **2** (triangles) in the absence (open symbols) and presence of heparin (filled symbols). Peptides (5 μM) in 10 mM Tris, 100 mM NaCl, pH 7.0, heparin (20 μg mL^{-1}).

disulfide linked peptide 2 also undergoes a significant increase in helicity upon heparin addition, although because of the ordering imposed by the disulfide bond, significantly more helicity is observed in the absence of heparin.

Fluorescence titrations of the peptides 1 and 2 with heparin show the emergence of a fluorescence emission band centered at 560 nm which is indicative of excimer formation between the N-terminal fluorophores (Figure 3). Over the course of the titration the excimer emission band reaches a maximum at 10 μg mL^{-1} heparin and then decreases with increasing heparin concentration (Figure 3, inset). This ratiometric fluorescence response upon heparin binding may be useful for heparin sensor design.[36,37]

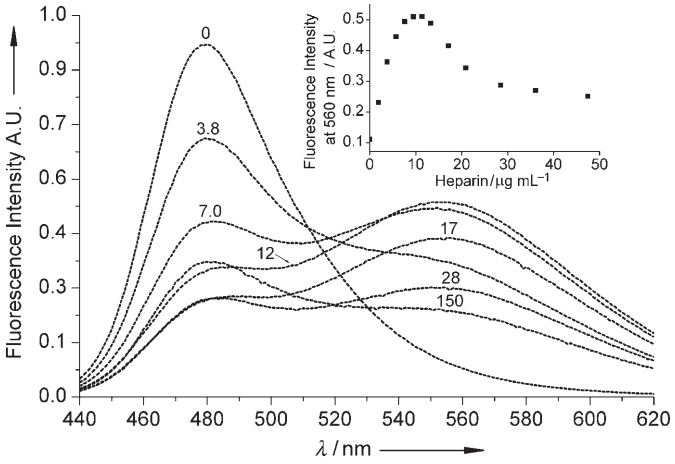

Figure 3. Fluorescence spectra of peptide 1 with increasing heparin concentrations. Peptide (5 μM), excitation 420 nm, 10 mM Tris, 100 mM NaCl, pH 7.0, heparin concentrations are indicated in μg mL^{-1} above the respective spectra.

Detailed analysis of the complex formation with the two peptides was carried out using fluorescence titrations with a purified dodecasaccharide heparin fragment. The use of the defined heparin fragment allowed accurate concentrations to be used and allowed analysis by analytical ultracentrifugation. These titrations of the dodeccasaccharide were fit to the model proposed in Figure 4 (see also the Supporting Information). Titrations of peptide 1 carried out at low peptide concentrations (low nM) do not show any excimer emission but do display fluorescence quenching with increasing heparin concentrations. This binding event is fit to a 1:1 peptide–heparin complex formation (I). At higher peptide concentrations the excimer fluorescence is observed. The excimer signal increases in the titration to a maximum and then decreases in the presence of excess polysaccharide, as was observed in titrations with unfractionated heparin. This behavior was assigned to be the formation of a 1:2 heparin–coiled-coil species (II) with a maximum excimer emission and upon excess heparin addition a 2:2 (III) species with a lower excimer quantum yield. Global fitting the titration data to these three equilibria gave the dissociation constants shown in Figure 4. The equilibria between the 1:2 complex (II) and 2:2 complex (III) was determined under conditions of excess heparin dodecasaccharide at varied peptide concentrations.

Similarly, titration of the disulfide linked peptide 2 gave a strong excimer signal with increasing heparin concentrations at all peptide concentrations investigated (100 nM–10 μM). The excimer signal also reached a maximum and decreased with excess heparin. Global fitting of the fluorescence titration data gave dissociation constants of 16 nM and 2 μM for the first and second heparin binding events.

Throughout the heparin titrations of peptides 1 and 2 no dependence on time was observed and the fluorescence spectra were stable for days. Attempts at thermal denaturation experiments lead to aggregation of the peptide samples in the presence of heparin.

Sedimentation equilibrium analysis of solutions containing species III and V were carried out, as at high peptide and heparin concentrations the equilibria allows solutions containing predominantly these complexes to be obtained. The molecular weights calculated using a weight averaged v-bar value for the heparin dodecamer peptide 2:2 complexes gave masses in close approximation (±15%) to those expected. The deviation in the molecular weights is likely due to the estimations necessary in the v-bar calculation of the complex.

The selectivity of the complex formation was explored by titrating peptides 1 and 2 with other biologically relevant polyanions: chondroitin sulfate A, DNA, and polyglutamic acid. To monitor the complex formation the ratio of fluorescence at 477 nm and 560 nm was used as the signature of excimer fluorescence observed in the heparin peptide complexes. As would be expected, the strongly cationic peptides did associate with the other polyanions as was evident by the changes in fluorescence emission of the peptides during the course of the titrations. However, the same intensity of excimer fluores-

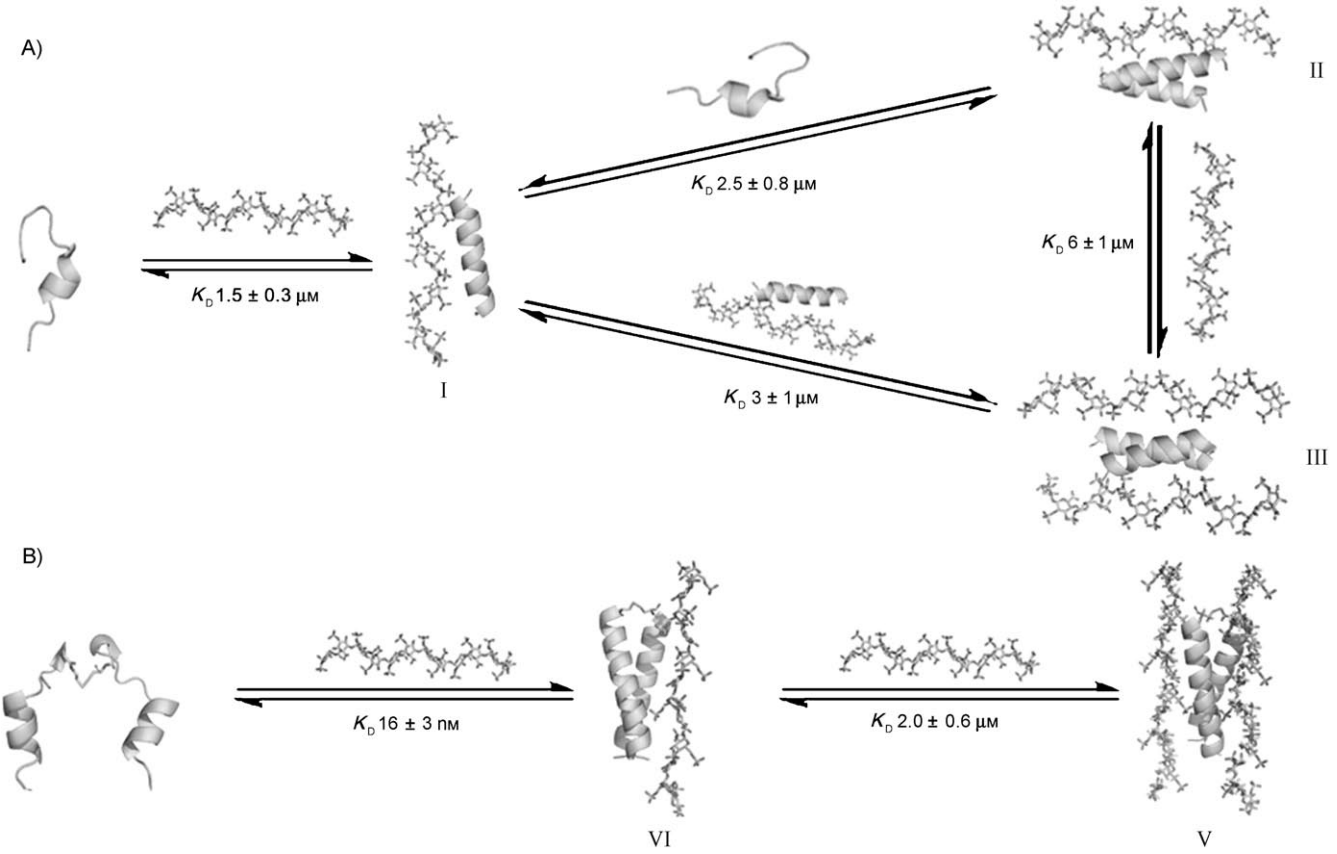

Figure 4. Proposed equilibrium binding model of heparin dodecamer and engineered peptides A) peptide **1**; B) disulfide linked peptide **2**. All binding studies were carried out in 10 mM Tris, 100 mM NaCl, pH 7.0

cence signal was not observed with these other polyanions (Figure 5), suggesting that the heparin complex is structurally distinct from the complexes formed from the other polyanions.

In conclusion we have shown that the strong electrostatic interaction observed during heparin binding can be harnessed to drive the change in conformation and defined oligomerization of a designed peptide. In the system described herein the peptide conformational change is coupled to a ratiometric fluorescence response which will be explored as a sensor of heparin or the S domains of heparan. This example of using heparin to order peptide structures may be useful for protein engineering, nanotechnology, and in furthering the understanding of glycosaminoglycan protein interactions.

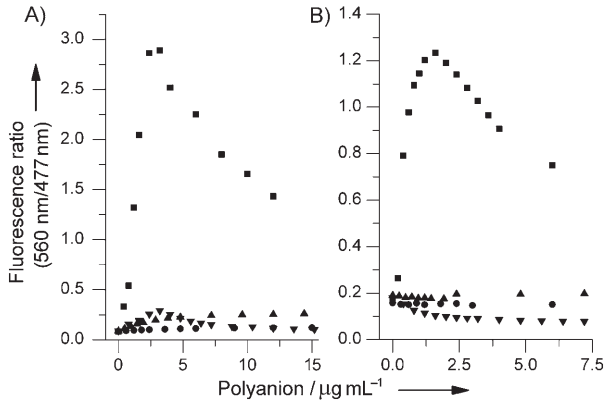

Figure 5. Selectivity of coiled-coil peptides. A) Peptide 1 (2 μM) or B) Disulfide linked peptide **2** (500 nM) were titrated with heparin (■), DNA (▼), chondroitin sulfate A (▲), or polyglutamic acid (●) in phosphate buffered saline (pH 7.0).

Experimental Section

General: All peptides were synthesized by standard Fmoc solid phase peptide synthesis. 7-Diethylaminocoumarin-3-carboxylic acid was prepared by literature methods and coupled to the N terminus of the peptides using standard conditions.[38] Heparinase I was purchased from IBEX pharmaceuticals. Unfractionated heparin (164 U mg^{-1}) from porcine mucosa was purchased from Celsius laboratories and oligosaccharide fractions of heparin were produced by depolymerizing heparin with Heparinase I and fractionating with GPC as previously reported.[39] A range of sulfation patterns is present in this homogenous length fraction and on average 2–3 sulfate groups per disaccharide were observed by ESI-MS. All experimental details can be found in the Supporting Information.

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Keywords: coiled-coil · fluorescence · glycosaminoglycan · heparin · peptides

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