

Interaction of Heparin with Two Synthetic Peptides that Neutralize the Anticoagulant Activity of Heparin[†]

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ABSTRACT: Two synthetic analogues of the heparin-binding domain of heparin/heparan sulfate-interacting protein (Ac-SRGKAKVKAKVKDQTK-NH₂) and the all-D-amino acid version of the same peptide (L-HIPAP and D-HIPAP, respectively) were synthesized, and their efficacy as agents for neutralization of the anticoagulant activity of heparin was assayed. The two analogue peptides were found to be equally effective for neutralization of the anticoagulant activity of heparin, as measured by restoration of the activity of serine protease factor Xa by the Coatest heparin method. The finding that L-HIPAP and D-HIPAP are equally effective suggests that D-amino acid peptides show promise as proteolytically stable therapeutic agents for neutralization of the anticoagulant activity of heparin. The interaction of L-HIPAP and D-HIPAP with heparin was characterized by ¹H NMR, isothermal titration calorimetry (ITC), and heparin affinity chromatography. The two peptides were found to interact identically with heparin. Analysis of the dependence of heparin–peptide binding constants on Na⁺ concentration by counterion condensation theory indicates that, on average, 2.35 Na⁺ ions are displaced from heparin per peptide molecule bound and one peptide molecule binds per hexasaccharide segment of heparin. The analysis also indicates that both ionic and nonionic interactions contribute to the binding constant, with the ionic contribution decreasing as the Na⁺ concentration increases.

Heparin, a linear polysaccharide comprised of highly sulfated 1 → 4 linked uronic acid-(1→4)-D-glucosamine repeating disaccharide units, has been used clinically as an anticoagulant for more than half a century (1, 2). Other biological activities include release of lipoprotein lipase and hepatic lipase (3, 4), regulation of complement activation (5), regulation of angiogenesis and tumor growth (6–8), regulation of Burkitt's lymphoma growth (9), modulation of inflammation (10), and antiviral activity (11–15). The biological activity of heparin results from its interaction with proteins, and hundreds of proteins have been shown to interact with heparin (2, 16–18).

Heparin functions as an anticoagulant by binding to antithrombin III (AT),¹ a serine protease inhibitor that mediates the coagulation cascade; binding causes a conformational change that accelerates inhibition of serine protease factor Xa by AT (19). In some clinical situations, for example, where extracorporeal blood circulation is necessary

or when patients experience moderate to severe bleeding, the anticoagulant activity must be neutralized to reduce the risk of hemorrhagic hazard. Protamine, a complex mixture of arginine-rich peptides, is used for this purpose. However, protamine can induce life-threatening side effects, such as hypotension and anaphylactoid-like symptoms, including bradycardia, dyspnea, and flushing (20–24). Thus, there is interest in developing alternatives to protamine.

Carson and co-workers have shown that a cell surface heparin–heparan sulfate-interacting protein (HIP) found in a variety of human epithelial and endothelial cells binds to heparin (25). The heparin-binding domain was found to have the sequence CRPKAKAKAKDQTK (26). A synthetic peptide of the same sequence (HIP peptide) competes with AT for heparin and neutralizes the anticoagulant activity of heparin in blood plasma assays (26), which suggests that HIP peptide or peptides with similar sequences may have potential as clinical agents for neutralization of the anticoagulant activity of heparin (25–28).

In this paper, we report on the interaction of heparin with two synthetic analogues of HIP peptide: Ac-SRGKAKVKAKVKDQTK-NH₂ and the same peptide synthesized with all D-amino acids, known as L-HIP analogue peptide (L-HIPAP) and D-HIPAP, respectively. Compared to the HIP peptide, the N-terminus has been acetylated and the C-terminus amidated to eliminate end group effects, the cysteine residue has been substituted with serine to eliminate dimerization by disulfide bond formation, the proline has been substituted with glycine to eliminate cis–trans isomerism across the arginine–proline peptide bond, and two alanine residues have been replaced with valine to facilitate characterization of the

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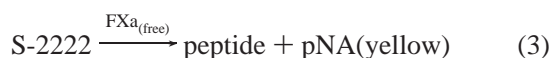
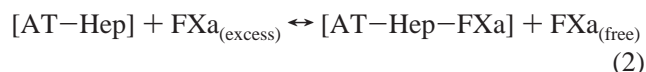
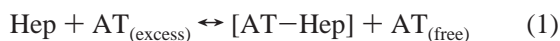
¹ Abbreviations: AT, antithrombin III; HIP, heparin/heparan sulfate-interacting protein; HIP peptide, CRPKAKAKAKDQTK; L-HIPAP and D-HIPAP, Ac-SRGKAKVKAKVKDQTK-NH₂ synthesized from all L- and all D-amino acids, respectively; Hep, heparin; FXa, factor Xa; TOCSY, total correlation spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; BASHD-TOCSY, band-selective homonuclear-decoupled TOCSY; ITC, isothermal titration calorimetry.

interaction of the peptides with heparin by NMR. Because polypeptides are rapidly degraded by proteases *in vivo* and thus are generally poor drugs, both the all-L-amino acid and all-D-amino acid peptides were studied. Both L-HIPAP and D-HIPAP were found to be effective agents, and to be equally effective, for neutralization of the anticoagulant activity of heparin, as determined by the Coatest heparin method (26). Interaction of both peptides with heparin was studied by ^1H NMR and heparin affinity chromatography, and heparin–peptide binding constants were determined by isothermal titration calorimetry.

MATERIALS AND METHODS

Chemicals. L-HIPAP and D-HIPAP were synthesized as described below. Porcine intestinal mucosal heparin (sodium salt) was obtained from Sigma Chemical Co., D_2O (99.8%) from Isotec, and sodium 3-(trimethylsilyl)propionate-2,2,3,3- d_4 (TMSP) from Cambridge Isotope Labs. All other chemicals were reagent grade. The Coatest heparin assay kit was obtained from DiaPharma.

Anticoagulation assays were performed using the Coatest heparin method for determination of the level of heparin in blood plasma. The Coatest method is based on the following reactions:



where FXa represents factor Xa and S-2222 represents Bz-Ile-Glu-(γ -OR)-Gly-Arg-pNA, a chromogenic substrate cleaved by free FXa to peptide Bz-Ile-Glu-(γ -OR)-Gly-Arg and pNA (*p*-nitroaniline). Heparin binds to AT, causing a change in the conformation of AT to a form that binds to and sequesters the activity of FXa. Addition of a peptide that competes with AT for heparin will increase the concentration of free FXa. Thus, restoration of the activity of FXa, as indicated by the amount of S-2222 converted to peptide and pNA, provides a measure of the ability of a peptide to neutralize the anticoagulant activity of heparin (26).

The assay method was calibrated using standard solutions that contained 0.1 IU/mL AT and heparin at concentrations ranging from 0.01 to 0.07 IU/mL in buffer and normal human plasma supplied with the Coatest kit. The standard solutions were incubated at 37 °C for 3–4 min, after which 100 μL of bovine factor Xa (0.71 nkat) was added to 200 μL of each standard solution. The mixtures were incubated at 37 °C for 30 s, after which 200 μL of S-2222 (0.2 μmol , 37 °C) was added, and the reaction mixtures were incubated at 37 °C for exactly 3 min. Reaction was stopped by addition of 300 μL of 20% (v/v) acetic acid. The absorbance was measured at 405 nm to quantitate the formation of pNA. The ability of L-HIPAP and D-HIPAP to neutralize the anticoagulant activity of heparin was determined by addition of a range of concentrations of each peptide to the 0.07 IU/mL standard heparin solution, and the assay was performed as described

above. Triplicate experiments were performed in the absence of peptide and at each peptide concentration.

Peptide Synthesis. L-HIPAP and D-HIPAP were synthesized on a Millipore model 9050 plus solid phase peptide synthesizer using Fmoc peptide synthesis methodology (29). Fmoc-PAL-PEG-PS resin with a loading capacity of 0.20 mmol/g was conditioned by immersion in *N,N*-dimethylformamide (Fisher Scientific) for 1 h. Side chain-protected Fmoc amino acids were obtained from Chem-Impex International and Nova Biochem. The α -carboxyl groups of the incoming amino acids were activated with 1-hydroxy-7-azabenzotriazole (HOAt, Applied Biosystems); the Fmoc protecting group on the last amino acid added was removed with 20% piperidine in *N,N*-dimethylformamide, and the activated α -carboxyl group and the deprotected amino group were coupled by reaction for 1 h. The N-terminal amino group was acetylated with acetic anhydride. Side chain functional groups were deprotected, and the peptide was cleaved from the resin by reaction with a cleavage cocktail composed of 88% trifluoroacetic acid (TFA), 5.8% phenol, 2% triisopropylsilane, and 4.2% water for 4 h. The cleaved and deprotected peptide mixture was lyophilized and redissolved in water, and the peptide was isolated by reverse-phase HPLC on a semi-prep scale C18 column. The peak corresponding to the desired peptide in the chromatogram was identified by MALDI-TOF mass spectrometry.

NMR Measurements. NMR spectra were measured on a Varian Inova 500 MHz spectrometer equipped with wave-form generators, a Performa X, Y, Z gradient module, and a $^1\text{H}\{^{13}\text{C},^{15}\text{N}\}$ triple-resonance, X, Y, Z triple-axis pulsed field gradient probe. One-dimensional ^1H NMR spectra and two-dimensional TOCSY, ROESY, and NOESY and BASHD (band-selective homonuclear decoupled)-TOCSY, ROESY, and NOESY spectra (30–35) were recorded by standard pulse sequences with suppression of the water resonance by presaturation. BASHD experiments were conducted with band selection of both the NH and C_αH regions (33–35).

NMR samples were prepared in a 90% H_2O /10% D_2O mixture. The peptide concentration was generally 5 mM and the heparin concentration 20 mM in terms of the sulfated uronic acid-(1 \rightarrow 4)-glucosamine repeating disaccharide unit. TMSP was added as the chemical shift reference. Shigemi tubes were used for all NMR experiments; the sample volume was 300 μL .

Circular Dichroism Measurements. CD spectra were recorded for pH 5.5 solutions of heparin, the peptide, and heparin and peptide with a Jasco J-600 spectrophotometer. The concentrations of peptide solutions were 0.1 mM; the concentration of the heparin solution was 0.4 mM (in terms of the concentration of the major repeating disaccharide unit), and the peptide and heparin concentrations of the heparin/peptide solutions were 0.1 and 0.4 mM, respectively.

Isothermal Titration Calorimetry. ITC experiments were performed at 25 °C using a MicroCal VP-ITC microcalorimeter (36, 37). Heparin and peptide solutions were prepared in 50 mM phosphate buffer (pH 7.0) at different concentrations of NaCl. The heparin solutions ranged in concentration from 0.68 to 0.092 mM, in terms of an average molecular mass of 12 000 Da, and the peptide solutions ranged in concentration from 0.15 to 0.18 mM. The peptide solution was placed in the calorimeter cell, and the heparin solution was loaded into the syringe injector. The titrations involved

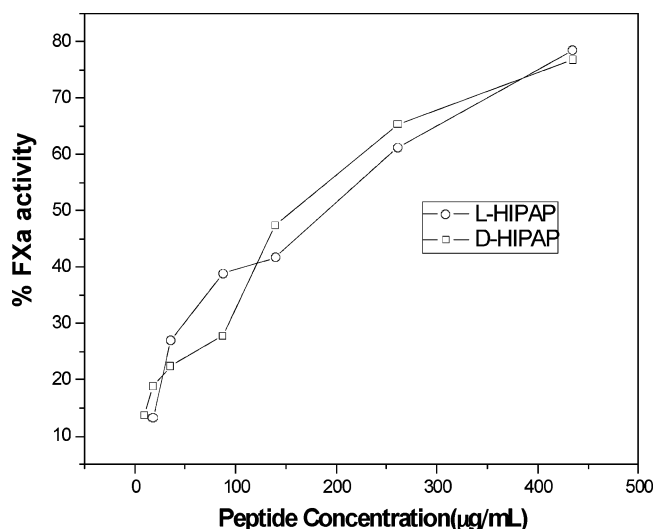


FIGURE 1: Restoration of the activity of FXa as a function of the concentration of L-HIPAP and D-HIPAP.

the injection of 5 μ L of heparin solution into the peptide solution, with a 250 s delay between injections. The ITC unit measured the heat absorbed due to interaction of heparin with the peptide following the addition of each aliquot of heparin. Calorimetric titration data were fit to give the stoichiometry (N), the association constant (K), and the binding enthalpy (ΔH), using the Origin 5.0 nonlinear least-squares program supplied with the Microcal VP-ITC instrument. The reported binding constants are the average of duplicate measurements. The effect of dilution of the heparin solution in the titration cell was removed by subtracting calorimetric data for a blank titration, which consisted of titration of heparin into buffer solution.

Affinity Chromatography. Affinity chromatography experiments were performed with a Dionex 500 HPLC system using a HiTrap heparin HP column (0.7 cm \times 2.5 cm, 1.0 mL column volume). Peptide solutions were prepared in 5 mM phosphate buffer (pH 7.0) and were eluted using a linear gradient of low-salt and high-salt buffers [5 mM phosphate (pH 7.0) and 5 mM phosphate (pH 7.0) with 1.0 M NaCl, respectively] at a flow rate of 0.6 mL/min with a 65 min gradient reaching 65% high-salt buffer.

RESULTS

Neutralization of the Anticoagulant Activity of Heparin. The effect of L-HIPAP and D-HIPAP on the anticoagulant activity of heparin was determined using the Coatest heparin method. The absorbance of pNA for the standard heparin solutions decreased linearly as the heparin concentration increased, confirming that the FXa activity is proportional to the amount of pNA formed. The results in Figure 1, presented in terms of the % FXa activity, indicate that both L-HIPAP and D-HIPAP restore FXa activity in a concentration-dependent manner and, most importantly, that they are equally effective in restoring activity. In view of these findings, we have characterized in detail the interaction of L-HIPAP and D-HIPAP with heparin by ^1H NMR and isothermal titration calorimetry and heparin affinity chromatography.

^1H NMR Studies of the Interaction of L-HIPAP and D-HIPAP with Heparin. To determine the appropriate condi-

tions for characterization of the interaction of L-HIPAP and D-HIPAP with heparin by NMR, spectra were measured at heparin-to-peptide ratios ranging from 0:1 to 5:1, where the heparin concentration is expressed in terms of the repeating heparin disaccharide unit. The spectrum was measured for a 5 mM solution of L-HIPAP at pH 5.1. Heparin was then added directly to the NMR tube to increase the ratio in increments of 0.5:1, the pH adjusted to between 5.0 and 6.0, and the spectrum measured again until a ratio of 5:1 was reached. At a ratio of 0.5:1, the solution was cloudy. At this ratio, essentially all the heparin is bound to peptide (as indicated by the ITC results that follow), while the peptide exists both free and bound to heparin. The backbone amide NH region of the NMR spectrum shows most of the features seen before the addition of heparin, but the resonances are broadened. At a ratio of 1:1, more peptide is bound and there is extensive broadening of the resonances. At a ratio of 2:1, the solution was clear and the resonances were sharper. The spectrum changed little, and the solution remained clear as the ratio was increased to 4:1. At a ratio of >4:1, there was no further change in the spectrum, indicating that essentially all the peptide was in a bound form. The resonances of heparin-bound peptide are broader than those of the free peptide, presumably because the bound peptide has the motional properties of heparin and thus its resonances have shorter relaxation times.

The backbone amide NH resonances of free and heparin-complexed peptides were assigned to amino acid type using scalar connectivities to side chain protons in BASHD-TOCSY spectra. Then the resonances were assigned to specific amino acids in the peptide sequence using dipolar cross-peaks in the $\text{C}_\alpha\text{H-NH}$ region of BASHD-ROESY spectra for the free peptides or BASHD-NOESY spectra for the heparin-complexed peptides (33–35). Because of the multiple lysines and alanines in the peptides, there is extensive overlap of cross-peaks in their two-dimensional TOCSY, ROESY, and NOESY spectra. With the increased resolution in two-dimensional BASHD-TOCSY, ROESY, and NOESY spectra, it was possible to completely assign their ^1H NMR spectra; resolution is increased in the BASHD spectra as a result of collapse of ^1H spin-coupled multiplets to singlets in the indirectly detected (band-selected) F_1 dimension. To resolve all of the cross-peaks, it was necessary to measure BASHD-TOCSY and BASHD-ROESY spectra with band selection of the C_αH and the NH regions (34, 35).

The chemical shifts of resonances for all the protons in each peptide were obtained from BASHD-TOCSY subspectra. The chemical shifts of the backbone amide NH protons of free and heparin-complexed L-HIPAP are presented in Table 1. The chemical shifts of the corresponding protons of D-HIPAP are identical to those reported in Table 1.

Effect of Heparin on Titration of the Lysine Ammonium Groups. The interaction of heparin with L-HIPAP and D-HIPAP is expected to involve, in part at least, electrostatic interactions between the lysine ammonium and arginine guanidinium groups of the peptides and anionic sites on heparin. If so, the apparent pK_a values of the lysine ammonium groups that interact with heparin will be increased (38). pK_a values can be determined for each lysine ammonium group in a peptide from the pH dependence of the chemical shifts of the side chain $\text{C}_\epsilon\text{H}_2$ protons (39). Due to broadening of resonances for the heparin-complexed pep-

Table 1: Chemical Shifts of the Backbone Amide NH Resonances of L-HIPAP and the Heparin Complex of L-HIPAP

residue	chemical shift		$\Delta\delta^a$
	free peptide	peptide with heparin	
Ser ¹	8.352	8.336	0.016
Arg ²	8.512	8.438	0.074
Gly ³	8.398	8.376	0.022
Lys ⁴	8.164	8.108	0.056
Ala ⁵	8.278	8.268	0.010
Lys ⁶	8.366	8.154	0.212
Val ⁷	8.137	8.132	0.005
Lys ⁸	8.443	8.286	0.157
Ala ⁹	8.310	8.248	0.062
Lys ¹⁰	8.391	8.184	0.207
Val ¹¹	8.176	8.157	0.019
Lys ¹²	8.434	8.349	0.085
Asp ¹³	8.345	8.346	-0.001
Gln ¹⁴	8.542	8.467	0.075
Thr ¹⁵	8.330	8.327	0.003
Lys ¹⁶	8.256	8.108	0.148

$$^a \Delta\delta = \delta_{\text{free peptide}} - \delta_{\text{with heparin}}$$

tides, resonances for the C_αH₂ protons of each of the six lysines of heparin-complexed L-HIPAP could not be resolved. However, the C_αH₂ resonances for the six lysines shift together as their ammonium groups are titrated, indicating that all six lysine ammonium groups interact with heparin to some extent. Thus, the effect of binding on titration of the six lysine ammonium groups was established using the chemical shift of the composite C_αH₂ resonance. Composite chemical shift–pH data for free L-HIPAP and L-HIPAP with heparin are presented in Figure 2A. The titration curve is shifted to higher pH in the presence of heparin, consistent with interaction of the lysine ammonium groups with heparin.

The resonance for the C_δH₂ protons of the Arg residue also is shifted in the presence of heparin (Figure 2B), indicating interaction of the guanidinium group of arginine with heparin. At pH 11, where the lysine ammonium groups are essentially completely titrated to the neutral amino form, the resonance for the C_δH₂ protons of arginine is still displaced in the presence of heparin, indicating that the guanidinium group of arginine still interacts with heparin. At pH >11, the chemical shift of the C_δH₂ resonance shifts back to that of the free peptide due to the competitive binding of Na⁺ from the added NaOH to heparin and, starting at pH ~12, titration of the guanidinium group.

Resonances for the NH protons also provide evidence for interaction of the lysine ammonium and arginine guanidinium groups with heparin. The lysine ammonium and arginine guanidinium protons of the free peptide give broad resonances at low pH but disappear due to fast exchange with solvent protons at pH ~4 and ~5, respectively, in spectra measured as a function of increasing pH. These resonances are present in spectra measured at pH >5 and >6, respectively, in the presence of heparin, indicating that the ammonium and guanidinium protons are shielded from exchange by interaction of the ammonium and guanidinium groups with heparin.

Conformational Properties of L-HIPAP. The conformation of L-HIPAP free and complexed by heparin was investigated using the nuclear Overhauser effect (NOE) (40). The free peptide is an ensemble of rapidly interconverting random coil conformations, as indicated by the absence of NOEs

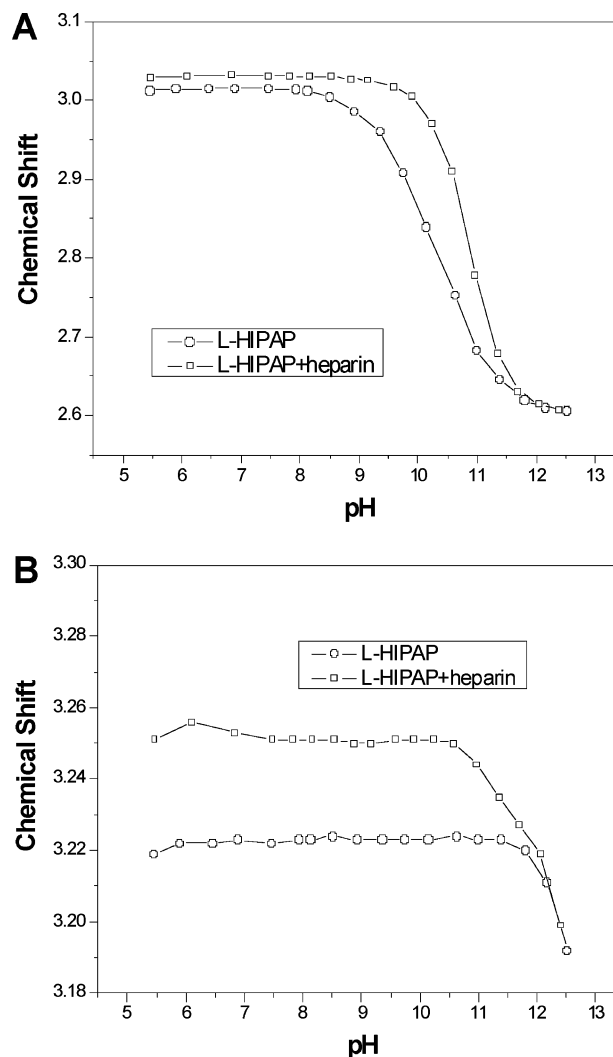


FIGURE 2: (A) Composite chemical shift–pH titration curves for the lysine C_αH₂ protons and (B) chemical shift–pH data for the arginine C_δH₂ protons of L-HIPAP in the absence and presence of heparin. The circles depict data for the free peptide, and squares depict data for the peptide with heparin, both at 25 °C. The peptide concentration was 5 mM, and the heparin disaccharide:peptide ratio was 4:1.

characteristic of peptides in a helical or β -strand secondary structure in two-dimensional ROESY spectra of the free peptide, including cross-peaks between amide NH protons on adjacent residues [NN(*i*, *i* + 1)]. Likewise, patterns of NOEs characteristic of helical or β -strand secondary structures were not observed in two-dimensional NOESY spectra of the heparin-complexed peptide. However, sequential NN-(*i*, *i* + 1) NOEs were observed for the S¹–A⁵ and V¹¹–K¹⁶ segments and between A⁹ and K¹⁰ of the heparin-complexed peptide, which together with reduced ³J_{NH–C α H} coupling constants indicate that the heparin-complexed peptide exists in fewer, more highly populated, conformations. However, the NOE data were not sufficient to establish the conformation(s) of the heparin-complexed peptide.

The CD spectra in Figure 3 provide additional information about the conformations of free and heparin-complexed L-HIPAP and D-HIPAP. A helical secondary structure for L-HIPAP would exhibit strong negative ellipticities at 208 and 222 nm and a positive ellipticity near 190 nm, while a β -sheet secondary structure would exhibit a strong negative ellipticity near 215 nm and a positive ellipticity at 195 nm

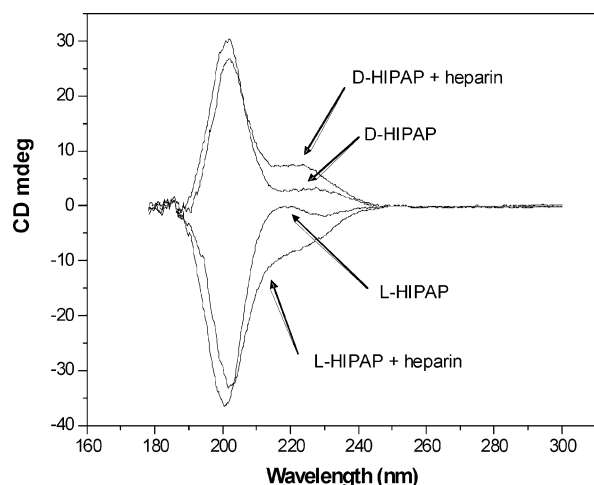


FIGURE 3: CD spectra of L-HIPAP and D-HIPAP, free and bound by heparin.

(41). The CD spectra change in the presence of heparin but do not exhibit these features, which is consistent with an ensemble of conformations. The CD spectra of L-HIPAP and D-HIPAP are essentially mirror images, the small differences between the CD spectra of the heparin-complexed peptides being due to the CD spectrum of heparin (see the Supporting Information).

Heparin–Peptide Binding Constants. Binding constants for the interaction of L-HIPAP and D-HIPAP with heparin were determined by isothermal titration calorimetry. A typical titration curve for L-HIPAP is shown in Figure 4. Binding constants were obtained by fitting the titration data to a model involving multiple equivalent binding sites. Results are reported in Table 2 for the binding of L-HIPAP by heparin as a function of the NaCl concentration. Results are also reported in Table 2 for the binding of D-HIPAP by heparin at NaCl concentrations of 0.0815 and 0.0915 M.

Affinity Chromatography. Peptides L-HIPAP and D-HIPAP were eluted from the heparin affinity column with essentially identical retention times (44.54 and 44.38 min, respectively).

DISCUSSION

Both L-HIPAP and D-HIPAP restore the activity of FXa. Because L-amino acid peptides are degraded by enzyme-catalyzed proteolysis, the finding that L-HIPAP and D-HIPAP are equally effective is particularly significant. Restoration of the activity of FXa by the parent HIP peptide was also assayed by the Coatest heparin method (25). However, a direct quantitative comparison of the efficacy of the parent HIP peptide and the two HIP peptide analogues is not possible because different heparin preparations were used in the two studies.

The results in Figure 2 indicate that the lysine ammonium groups of the HIPAP peptides are involved in binding, as expected if binding includes electrostatic interactions between cationic sites on the peptides and anionic sites on heparin. The fact that the $C_{\alpha}H_2$ resonances for all six lysines shift together in the chemical shift–pH titration experiment indicates that all six lysine ammonium groups are involved in the interaction with heparin. The differences between the chemical shifts of the backbone amide NH protons of free and heparin-complexed L-HIPAP (Table 1) are plotted in

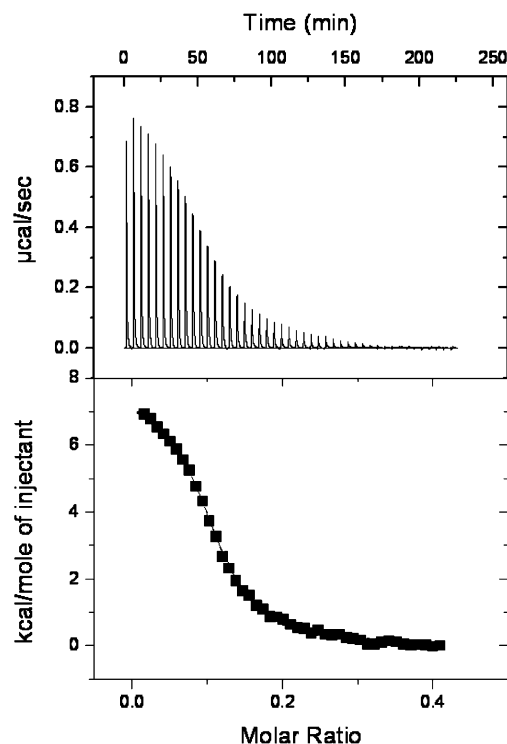


FIGURE 4: Binding isotherm for the interaction of L-HIPAP with heparin. The peptide (0.18 mM in the cell) was titrated with 0.57 mM heparin (in the syringe). In the top panel, the peaks indicate the heat absorbed after each addition of heparin. The peaks were integrated, and the total heat per injection (peak area) is plotted as a function of molar ratio in the bottom figure. The line through the points represents the fit of the ITC data that gave the thermodynamic parameters reported in Table 2.

Figure 5. The difference is largest for the lysines and arginine, consistent with their interaction with heparin.

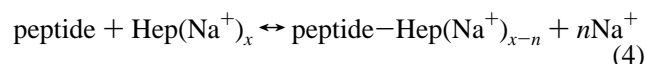
The findings that L-HIPAP and D-HIPAP are equally effective as agents for neutralization of the anticoagulant activity of heparin and that their heparin binding constants are identical suggest that it is the pattern, or spatial arrangement, of the lysine and arginine residues in L-HIPAP and D-HIPAP that is important in their interaction with heparin and not the recognition of a complementary structure by heparin. This conclusion is consistent with reports that the HIP peptide binds heparin by nonspecific charge interactions and that a peptide with a scrambled HIP peptide sequence (CKDKTPRAKQAAKK) had no effect on FXa activity when assayed by the Coatest heparin method (26, 42). The findings that the spatial arrangement of the six lysines and the one arginine is critical and that D-HIPAP is as effective as L-HIPAP should aid in the design of other HIP analogue peptides as potential therapeutic agents for neutralization of the anticoagulant activity of heparin.

The binding constants in Table 2 also provide information about the nature of the interaction of L-HIPAP and D-HIPAP with heparin. Heparin is a highly negatively charged polyelectrolyte, with a fraction of its negative charge neutralized by bound counterions (43). The counterion condensation model of polyelectrolyte binding developed by Manning (44, 45) predicts that the fraction of a Na^+ ion condensed per anionic charge on heparin (θ) is 0.59 (43). Experimental values of 0.58 and 0.63 have been reported (46, 47). Counterions are released when heparin binds to peptide, as

Table 2: Thermodynamic Data for the Interaction of L-HIPAP and D-HIPAP with Heparin at 25 °C

peptide	[Na ⁺] _{total} (M)	K_b (M ⁻¹)	N	ΔH (cal/mol)	ΔS (cal mol ⁻¹ °C ⁻¹)
L-HIPAP	0.0815	$(5.3 \pm 0.9) \times 10^4$	7.1 ± 0.5	$(9.6 \pm 0.8) \times 10^2$	24.7 ± 0.6
D-HIPAP	0.0815	$(4.5 \pm 0.6) \times 10^4$	7.9 ± 0.5	$(9.3 \pm 0.4) \times 10^2$	24.2 ± 0.4
L-HIPAP	0.0915	$(3.8 \pm 0.2) \times 10^4$	7.3 ± 0.1	$(9.7 \pm 0.1) \times 10^2$	24.1 ± 0.1
D-HIPAP	0.0915	$(3.8 \pm 0.2) \times 10^4$	7.3 ± 0.7	$(11.0 \pm 1) \times 10^2$	24.4 ± 0.5
L-HIPAP	0.107	$(2.3 \pm 0.6) \times 10^4$	6.7 ± 0.3	$(10.9 \pm 0.4) \times 10^2$	23.6 ± 0.3
L-HIPAP	0.132	$(1.5 \pm 0.6) \times 10^4$	6.7 ± 0.4	$(10.3 \pm 0.4) \times 10^2$	22.5 ± 0.7
L-HIPAP	0.182	$(5.9 \pm 0.1) \times 10^3$	7.7 ± 0.4	$(12.5 \pm 0.4) \times 10^2$	21.4 ± 0.1
L-HIPAP	0.232	$(5.0 \pm 0.3) \times 10^3$	6.1 ± 0.5	$(13.2 \pm 0.2) \times 10^2$	21.3 ± 0.8

described by the following binding equilibrium for sodium heparinate (38, 43, 48):



The number of Na⁺ ions displaced by peptide, n , is equal to $Z\psi$, where Z represents the number of ionic interactions the peptide makes with heparin and ψ is the apparent fraction of Na⁺ condensed per heparin anionic charge. ψ includes both the fraction of a Na⁺ ion condensed per heparin anionic charge (θ) and the screening effect of condensed Na⁺ ions on the interactions of residual heparin anionic charges. Thus, ψ is larger than θ ; a ψ of 0.8 has been reported for heparin (49).

Equation 4 predicts the extent of peptide binding will decrease as the Na⁺ concentration is increased, which was found for the binding of L-HIPAP (Table 2). The dependence of the binding constant on the Na⁺ concentration is given by the equation (48, 50, 51)

$$\log K_b = \log K_{\text{nonionic}} - Z\psi \log[\text{Na}^+] \quad (5)$$

where K_b is the measured binding constant and K_{nonionic} the contribution of nonionic interactions to the binding constant. A plot of $\log K_b$ versus $\log[\text{Na}^+]$ is linear, with a slope of -2.35 and an intercept of 2.12 (see the Supporting Information).

The slope indicates that, on average, 2.35 Na⁺ ions are displaced per peptide interaction. Using the value of 0.8 for ψ , a value of 2.94 is obtained for Z ; i.e., L-HIPAP makes on average ~ 3 ionic interactions with heparin that result in the displacement of 2.35 bound Na⁺ ions.

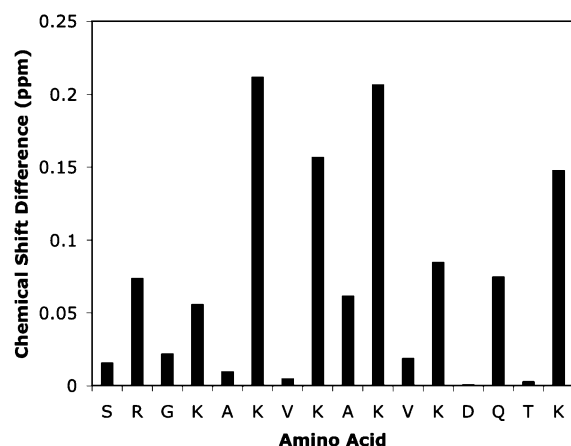


FIGURE 5: Change in the chemical shift of the backbone amide NH resonances of L-HIPAP upon binding to heparin. The chemical shift difference = $\delta_{\text{free peptide}} - \delta_{\text{with heparin}}$.

The binding constant is strongly dependent on the concentration of Na⁺, decreasing from 5.3×10^4 to 5.0×10^3 as the concentration of Na⁺ is increased from 0.082 to 0.232 M (Table 2). Both nonionic and ionic interactions contribute to the binding:

$$K_b = K_{\text{nonionic}} K_{\text{ionic}} \quad (6)$$

The intercept of 2.12 gives a value of 131 for K_{nonionic} . Using this value, K_{ionic} is calculated to be 400 at a Na⁺ concentration of 0.082 M and 38 at a Na⁺ concentration of 0.232 M. K_{nonionic} and K_{ionic} contribute equally to K_b at a Na⁺ concentration of 0.016 M. The relative magnitudes of K_{nonionic} and K_{ionic} indicate that both ionic and nonionic interactions, e.g., hydrogen bonding or hydrophobic interactions, make a significant contribution to the binding constant for the interaction of L-HIPAP with heparin. The relatively large displacement of the chemical shift of the glutamine NH resonance (Figure 5) suggests that hydrogen bonding to the side chain amide group of the glutamine residue is involved in binding to heparin.

The binding constants for L-HIPAP and D-HIPAP are identical at Na⁺ concentrations of 0.0815 and 0.0915 M (Table 2), and their elution times from the heparin affinity column are the same, both of which are consistent with the finding that L-HIPAP and D-HIPAP are equally effective at neutralizing the anticoagulant activity of heparin.

The average value of N , the number of peptides bound per heparin molecule, is 6.9 (Table 2), which corresponds to the binding of one peptide molecule per hexasaccharide segment of heparin. The thermodynamic parameters in Table 2 indicate that the binding reaction is entropically driven mainly by the release of ~ 3 Na⁺ ions per peptide bound. However, other interactions also contribute to the overall thermodynamics of the binding, including both ionic and nonionic interactions (enthalpic) and peptide conformational changes (entropic).

SUMMARY

The coagulation assay results indicate that L-HIPAP and D-HIPAP are equally effective agents for neutralization of the anticoagulant activity of heparin. NMR chemical shift data suggest that the two peptides interact identically with heparin and their binding constants, as determined by ITC, and their heparin affinity chromatography elution times are the same. The HIPAP peptides bind to heparin by a combination of ionic and nonionic interactions, with the ionic contribution decreasing as the Na⁺ counterion concentration increases. These results, taken together, suggest that D-amino acid peptides show promise as proteolytically stable thera-

peutic agents for neutralization of the anticoagulant activity of heparin.

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SUPPORTING INFORMATION AVAILABLE

CD spectra of the complexes and heparin (Figure 1) and a plot of $\log K_b$ versus $\log[\text{Na}^+]$ (Figure 2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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