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Interaction of heparin with synthetic antithrombin III peptide analogues

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Heparin-binding proteins may contain specific patterns of basic amino acids, called consensus sequences, that interact with heparin. Small peptides were synthesized that contained consensus sequences (i.e. FAKLNCRLYRKANKSSK) or disrupted consensus sequences (i.e. K136→A) based on the known sequence of antithrombin III (amino acid residues 123–139). These peptides were then examined in both competitive and non-competitive binding experiments using bioassays, fluorescence spectroscopy, affinity chromatography and n.m.r. spectroscopy. Both the consensus and disrupted-consensus peptide bound to heparin. Peptides with consensus sequences bound specifically to the pentasaccharide antithrombin III-binding site within heparin. In contrast, peptides with disrupted consensus sequences showed no specificity, binding to any sequence within heparin. Proton nuclear Overhauser enhancement spectroscopy demonstrated the proximity of leucine and tyrosine (within the consensus sequence) to the *N*-acetyl moiety found primarily within the pentasaccharide antithrombin III-binding site of heparin. This experiment confirmed the findings of the other techniques and helped to localize the binding sites in both peptides and heparin. A model is proposed for both specific and non-specific heparin interaction with consensus and disrupted-consensus peptides.

INTRODUCTION

Glycosaminoglycan heparin, a prototypic acidic polysaccharide, has been used clinically as an anticoagulant and antithrombotic agent for more than half a century (Linhardt, 1991). Over the past decade, heparin has been shown to bind to a myriad of different proteins (Linhardt and Loganathan, 1990) to mediate a variety of biological processes (Jaques, 1979) including coagulation (Rosenberg and Damus, 1973), angiogenesis (Folkman et al., 1983), complement activation (Edens et al., 1993), adhesion (Khan et al., 1988; Skubitz et al., 1988; Tschopp et al., 1988), smooth-muscle proliferation (Castellot et al., 1984, 1986), platelet aggregation (Salzman et al., 1980) and lipolysis (Engelberg, 1977; Merchant et al., 1986). The discovery of these new activities has made the elucidation of heparin–protein structure–activity relationships very important.

The best studied of the acidic oligosaccharide–protein interactions (Rosenberg and Lam, 1979; Lindahl et al., 1979) involves the binding of heparin to a peptide sequence within antithrombin III (ATIII). This binding leads to a conformational change in ATIII and is responsible for the catalytic activity of heparin in the coagulation cascade. The precise peptide sequence within ATIII at which heparin binds has not yet been established (Smith and Knauer, 1987). In contrast, the heparin oligosaccharide sequence that binds to ATIII is known and structure–activity relationship studies on this oligosaccharide sequence have been performed (Atha et al., 1984, 1987). Thus the heparin–ATIII interaction is currently the best system for study despite a rather weak binding affinity and the complicating feature of an induced conformational change.

Molecular-modelling studies of protein–glycosaminoglycan interaction by Cardin and Weintraub in 1989 predict that heparin-binding proteins contain specific patterns of basic amino acids (consensus sequences). This model has been examined and the peptide sequence requirements for interaction with heparin have been studied (Lellouch and Lansbury, 1992). These previous experiments do not localize consensus peptide (CP) binding to the specific oligosaccharide sequence that binds ATIII (Cardin and Weintraub, 1989; Lellouch and Lansbury, 1992). In the present study, the Cardin and Weintraub model was used to select a small portion of a larger linear domain within ATIII (Smith and Knauer, 1987; Sun and Chung, 1990) known to be important in the interaction of heparin with ATIII. The specificity of binding of small peptides, with consensus and disrupted consensus sequences, to ATIII–affinity-fractionated heparin was examined. Bioassays, fluorescence spectroscopy, affinity chromatography and n.m.r. spectroscopy were used in both competitive (with ATIII) and non-competitive binding experiments to examine in detail the binding of CPs to heparin.

EXPERIMENTAL

Materials

Porcine intestinal mucosal heparin (167 units/mg) was purchased from Celsus Laboratories, Cincinnati, OH, U.S.A. Reference standard heparin sodium K1 (335 units/ml) was from the USP, Rockville, MD, U.S.A. Purified ATIII was previously prepared in our laboratory (Lee, 1991). Heparin lyase I (heparinase I, EC 4.2.2.7) was prepared by fermentation of *Flavobacterium heparinum* and purified to homogeneity (Lohse and Linhardt, 1992). Dermatan sulphate, concanavalin A–Sepharose 6B, strong anionic-exchange resin (Dowex Macroporous Resin, MSA-2) and heparin insolubilized on acrylic beads were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. ATIII–agarose was from Cal-Biochem, La Jolla, CA, U.S.A. Spectra/Por dialysis tubing (M_r cut-off 3000) was from Spectrum Medical, Los Angeles, CA, U.S.A. Factor Xa amidolytic kit including S-2222 [benzoyl-Ilė-Glu(-OR)-Gly-Arg-*p*-nitroanilide hydrochloride] was obtained from Kabi Pharmacia, Franklin, OH, U.S.A. Protein assay reagent and fluoraldehyde protein/peptide assay reagent were from Pierce, Rockford, IL, U.S.A. Electrophoresis was performed on a SE 620 slab unit 1405 cell from Hoefer, San Francisco, CA, U.S.A. using a 1420B power supply from Bio-Rad, Hercules, CA, U.S.A. High-purity electrophoresis reagents

Abbreviations used: ATIII, antithrombin III; UFH, unfractionated heparin; LAH, low-ATIII affinity heparin; HAH, high-ATIII affinity heparin; CP, consensus peptide; nOe, nuclear Overhauser effect.

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were purchased from IBI, Los Angeles, CA, U.S.A. Spectrometric measurements were made on a UV-160 spectrophotometer from Shimadzu, Tokyo, Japan, a Shimadzu spectrofluorophotomer, WM 360 MHz and AMX 600 MHz spectrometers from Bruker, and a VG ZAB-HF mass spectrometer from VG Analytical Ltd., Manchester, U.K. 3-[2,2,3,3-^{2}H](trimethylsilyl)propionic acid (sodium salt), ^2H_2O (99.96 and 99.9 atom % ^{2}H), deuterium chloride (99 atom % ^{2}H) and sodium deuteroxide (99 atom % ^{2}H) were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Micro-centrifuge concentrators were from Amicon Co., Beverly, MA, U.S.A. Conductivity and pH were measured using a Solution Analyzer 4530A from Amber Science, San Diego, CA, U.S.A. A strong anion-exchange h.p.l.c. column (5 μm particle size; 4.6 mm × 250 mm) was purchased from Phase Separation, Norwalk, CT, U.S.A. and C18 μBondapak reversed-phase h.p.l.c. columns were from Waters, Milford, MA, U.S.A. *t*-Butyloxycarbonyl (t-Boc) amino acids were purchased from Advanced ChemTech., Louisville, KY, U.S.A. *p*-Methylbenzhydrylamine resin was from Colorado Biotechnology Associates, Denver, CO, U.S.A. and trifluoroacetic acid was from Halocarbon Products of Augusta, SC, U.S.A. All other reagents for peptide synthesis were from Fisher Scientific, Pittsburgh, PA, U.S.A. or Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Preparation and characterization of heparin with high (HAH) or low (LAH) affinity for ATIII

Approx. 5 g of heparin was dissolved in 100 ml of deionized distilled water, dialysed exhaustively (M_r cut-off 3000), freeze-dried and prepared as a stock solution at 20 mg/ml in deionized distilled water. Heparin was fractionated on the basis of its affinity for ATIII using adaptations of previously described methods (Denton et al., 1981; Kim, 1988). LAH and HAH were dialysed (M_r cut-off 3000), lyophilized and dissolved in 250 μl of deionized distilled water. Residual protein, measured using fluoraldehyde (Benson and Hare, 1980), was removed by passing LAH and HAH through a beaded acrylic heparin column.

The amount of heparin in each fraction was determined from the uronic acid content using the carbazole assay (Bitter and Muir, 1962). The M_r values of unfractionated heparin (UFH), LAH and HAH were all approx. 12000 as estimated by gradient PAGE (Edens et al., 1992). The ATIII-mediated antifactor Xa activity of affinity-fractionated heparin was measured against a standard curve prepared with USP standard heparin using a Heparin Coatest kit from Kabi Pharmacia. Oligosaccharide compositional analysis was performed using methods previously developed in our laboratory (Rice et al., 1985; Linhardt et al., 1988, 1990, 1992a; Mallis et al., 1989; Loganathan et al., 1990; Wang, 1992). Fractionated heparin (100 μg) was dissolved in 50 μl of 50 mM sodium phosphate buffer containing 50 mM NaCl at pH 7.4 and subjected to complete digestion by adding 2 munits of heparin lyase I (Lohse and Linhardt, 1992) and incubating at 30 °C overnight. Depolymerized affinity-fractionated heparin was analysed by discontinuous gradient PAGE (Linhardt et al., 1990).

Peptide synthesis and purification

CP, modified CP, disrupted CP, modified–disrupted CP and rearranged CP (see Table 1) were synthesized using the t-bag technique in which *p*-methylbenzhydrylamine resin was compartmentalized in polypropylene bags (Houghten, 1985; Houghten et al., 1986). The bags were pooled for deblocking, base washing and capping and were separated only for the coupling reactions. Amino acid side chains were protected as Arg (*N*-guanidinotosyl), Cys (*S*-acetamidomethyl), Lys (N^{ϵ}-2-chlorobenzyloxcarbonyl), Phe (*N*-acetyl) Ser (*O*-benzyl) and Tyr (*O*-2,6-dichlorobenzyl). The peptides were cleaved from the resin using HF, and their side chains deprotected without removing the resin from the bags (Houghten et al., 1986). As many as ten bags of resin were cleaved simultaneously using a standard HF/anisole procedure (Houghten et al., 1986) in a compartmentalized reaction vessel from Multiple Peptide Systems, San Diego, CA, U.S.A. Residual anisole was removed by adding ethyl acetate before extracting the peptides with 15% acetic acid. After lyophilization, the crude peptides were analysed by reversed-phase h.p.l.c. (Waters μBondapak C18; 3.9 mm × 300 mm) using linear gradients from a starting solution of 0.1% trifluoroacetic acid in water to 0.04% trifluoroacetic acid in acetonitrile. Preparative purification of these peptides was carried out using similar gradients and a Waters μBondaPak C18 column (19 mm × 150 mm).

Assessment of peptide identity and homogeneity

Fast-atom-bombardment m.s. was carried out by the High-Resolution Mass Spectrometry Facility in the Department of Chemistry at the University of Iowa. A ZAB HF VG analytical mass spectrometer was used to identify the M_r and to confirm the complete deprotection and purity of the peptides in a thioglycerol matrix. Peptides were sequenced using a glycerol/thioglycerol matrix by fast-atom bombardment m.s. analysis conducted by M-Scan (West Chester, PA, U.S.A.) on a VG Analytical ZAB 2-SE high-field mass spectrometer operating at 8 kV. A caesium ion source was used to generate ions, and mass spectra were recorded using a PDP 11-250J data system. Mass calibration was performed using caesium iodide. Lyophilized samples were submitted for amino acid analysis to the Protein Structure Facility in the Department of Biochemistry at the University of Iowa. After liquid-phase HCl hydrolysis, amino acids were quantified using a Beckman 6300 high-performance ion-exchange analyser which used a 12 cm hydrolysate column with post-column ninhydrin detection.

One- and two-dimensional n.m.r. spectroscopy were performed on MSL-300, AMX-600 or Bruker WM-360 spectrometers operating under ASPECT 2000 and 3000 control. Purified peptide sample (2–2.5 mg) was dissolved at 2 mM in ^2H_2O (> 99.996% ^{2}H) containing 5 mM sodium phosphate buffer, pH 7.0, and freeze-dried. The sample was freeze-dried twice more from ^2H_2O before reconstitution in 500 μl of ^2H_2O. Data were obtained by one-dimensional or two-dimensional ^{1}H-n.m.r. using correlation, nuclear Overhauser effect (nOe), heteronuclear multiple quantum coherence spectroscopy and homonuclear Hartmann–Hahn recorded at 37 °C (310 K) principally at 360 MHz. 3-[2,2,3,3,-^{2}H](Trimethylsilyl)propionic acid (sodium salt) was added as internal standard.

Competitive binding of ATIII and CPs to affinity-fractionated heparins measured by fluorescence

Fluorescence was measured by using a spectrofluorophotometer equipped with a thermostatically controlled cell. All samples were prepared by using 50 mM sodium phosphate buffer containing 50 mM NaCl, pH 7.4, and excited at 280 nm with a 5 nm bandwidth. Emission spectra were scanned at least four times from 310 nm to 500 nm at 20 nm bandwidth to improve the quality of the spectra. Readings were obtained at 340 nm and integrated over 20 s. To avoid rapid protein adsorption to the quartz cuvette, a siliconized 0.7 ml quartz cell was used. The

increase in fluorescence was expressed as $(F-F_0)/F_0$, where F is the fluorescence intensity of the ATIII sample containing affinity-fractionated heparin, and F_0 represents that of the ATIII sample containing no heparin. All measurements were performed in triplicate.

ATIII (2.8 μl, 0.73 mg/ml), stored on solid CO_2 in individual vials, was thawed immediately before being added to 700 μl of buffer or to 700 μl of buffer containing affinity-fractionated heparin. The intrinsic fluorescence of 50 nM ATIII was measured in triplicate using a 700 μl cuvette, and designated F_0. A 250 nM solution of heparin was prepared in 700 μl of buffer and combined with 2.8 μl of 0.73 mg/ml ATIII (50 nM). The heparin and ATIII were gently mixed and fluorescence was measured immediately in the thermostatically controlled cell at 25 °C. The fluorescence value obtained on saturation of ATIII with 250 nM of heparin (UFH, HAH and LAH) was designated F. Peptide (0–50 μl of 1 mM stock solution prepared in buffer) was added to this solution of heparin–ATIII complex. After being mixed well, the fluorescence was measured, integrated over 20 s and designated F'. The degree of dissociation of ATIII from the complex after the addition of peptide was calculated using the following formula:

$$\% \text{ binding} = \{1-[(F-F')/(F-F_0)]\} \times 100$$

Measurement of competitive binding of ATIII and CPs to affinity-fractionated heparins by bioassay

Two sets of plastic test tubes were prepared for this experiment. CP, disrupted CP and rearranged CP (0–10 μM) were prepared in 200 μl of 50 mM sodium phosphate buffer at pH 7.4 containing 50 mM NaCl. ATIII (20 μl, 66.3 pmol) and plasma (20 μl) were added to each tube. HAH was added to one set of tubes to a final concentration of 10 nM, and the same volume of water was added to the other set. The tubes were mixed and then incubated at 37 °C for 30 min, during which time the peptides could compete with ATIII for binding to HAH. Factor Xa (0.71 kcat in 100 μl, kept on ice) was added, and the test tubes were mixed and then incubated for exactly 30 s. Substrate S-2222 (0.2 μmol in 200 μl) was then added to each tube and incubated for exactly 3 min. The reaction was quenched by adding 300 μl of aq. 50 % acetic acid. The extent of amidolysis was quantified by measuring the absorbance at 405 nm. The degree of competition between ATIII and (or disrupted CP control) was plotted as peptide concentration against u.v. absorbance (corresponding to remaining factor Xa activity).

Measurement of the affinity of peptides for heparin by chromatography

Each peptide (0.4 μmol) was dissolved in 1 ml of 20 mM Tris/HCl buffer at pH 7.4, loaded on to a 1 ml mini-column of heparin–acrylamide (675 μg of heparin/ml) and eluted with 20 mM Tris/HCl buffer at pH 7.4 containing NaCl (0.01–1 M). The amount of peptide eluted was determined by measuring the absorbance at 275 nm (tyrosine $\epsilon_{275} = 1470$).

Binding studies using ^{1}H-n.m.r. and nOe

The ^{1}H-n.m.r. binding experiments were performed on a Bruker AMX 600 spectrometer at an operating ^{1}H frequency of 600 MHz as solutions in ^2H_2O (500 μl) at ambient temperature. Data acquisition and processing was performed on-line with an ASPECT X32 computer using standard Bruker software (UXNMR). For both one-dimensional and two-dimensional n.m.r. experiments, 2.5 mg of peptide was dissolved in ^2H_2O (99.9 % atom ^{2}H, 1 ml), filtered through a 0.2 μm membrane and lyophilized. This process was repeated once more with ^2H_2O (99.96 % atom ^{2}H). Two-dimensional experiments such as correlation and nOe spectroscopy and homonuclear Hartmann–Hahn were performed using standard Bruker pulse programs. Typically, 256 data points were acquired in the f1 dimension, 2K data points in the f2 dimension followed by processing using sine bell or phase-shifted sine bell over 1K × 1K data matrix. A $\tau_{mix.}$ of 100–120 ms for homonuclear Hartmann–Hahn and 200 ms for nOe spectroscopy was used. Quadrature detection in the f1 dimension was employed.

Equimolar complexes of CP and disrupted CP with HAH were prepared by gradual addition of peptides to a dilute solution of HAH (100 μg in 1 ml). The steady-state nOe difference experiments were performed on these complexes at 298 K using standard Bruker software. Low-power 'soft' decoupling pulses for 7–9 s were used for complete population inversion and nOe transfer. Irradiation at a remote frequency served as a control. The difference spectra were generated by subtraction of the Fourier-transferred spectra. A minimum of 8000 scans was used to observe significant nOe.

RESULTS

Preparation and characterization of heparin, ATIII and peptides

UFH was fractionated into LAH (elution at 0.05 M salt) and HAH (elution at $\geq$ 1 M salt) using ATIII immobilized on concanavalin A–Sepharose. Analysis by a fluoraldehyde protein/peptide assay showed that both LAH and HAH were contaminated presumably with protein leaching from the affinity matrix. Both fractions were further purified by binding and release from a strong anion-exchange resin and by passage through a heparin–Sepharose column. The anti-(factor Xa) activities of LAH, HAH and UFH were 30, 635 and 167 units/mg respectively (using a standard curve prepared from USP heparin). Oligosaccharide maps (Linhardt et al., 1988, 1992a) were prepared by treating each heparin with heparin lyase I and analysing the oligosaccharide products by gradient PAGE and strong anion-exchange h.p.l.c. (not shown). The maps of HAH and LAH showed enrichment and 'disenrichment' respectively of oligosaccharide containing 3-*O*-sulphated glucosamine residue, corresponding to the heparin ATIII-binding site (Linhardt et al., 1992a,b). Human ATIII was purified to apparent homogeneity by SDS/PAGE, and its anti-(factor IIa) and anti-(factor Xa) activities were confirmed (Kim et al., 1988).

Peptides (Table 1) were prepared with their C-termini blocked as amides and the N-termini acetylated. To mimic the structure of ATIII, in which Cys-128 is disulphide-bonded with Cys-8, the

Table 1 Synthetic peptides studied

F is blocked Phe-*N*-acetyl residue, C-terminal K is blocked as amide and C is blocked with an acetamidomethyl group. CP is based on the ATIII sequence 123–139. The XBBBXXBX sequence (130–137) shown in parentheses represents the putative heparin-binding site in ATIII.

Peptide	Sequence
CP	FAKLNCRLYRKANKSSK (XBBBXXBX)
Modified CP (Y131→A)	FAKLNCRLARKANKSSK
Disrupted CP (K136→A)	FAKLNCRLYRKANASSK
Modified–disrupted CP (Y131 and K136→A)	FAKLNCRLARKANASSK
Rearranged CP (rearrangement)	FKAKNCRLYRAKSSNLK

Table 2 Assignment of the ^{1}H-n.m.r. resonances of CP and disrupted CP

No.	Residue	Proton	CP	Disrupted CP
123	Phe	α	4.55	4.56
		β	2.88	2.92
		β′	3.09	3.08
		Ar-H	7.25–7.40	7.25–7.40
124	Ala	α	4.33	4.34
		β	1.39	1.39
125	Lys	α	4.30	4.28
		β	1.84	1.86
		γ	1.42	1.46
		δ	1.72	1.76
		ε	2.95	3.00
126	Leu	α	4.27	4.34
		β	1.60	1.66
		γ	1.46	1.64
		δ	0.89	0.89
127	Asn	α	4.71	4.71
		β	2.76	2.75
		β′	2.85	2.85
128	Cys	α	4.52	4.53
		β	3.02	3.06
		β′	3.15	3.12
129	Arg	α	4.25	4.26
		β	1.78	1.84
		γ	1.57	1.64
		δ	3.22	3.18
130	Leu	α	4.28	4.34
		β	1.60	1.66
		γ	1.46	1.64
		δ	0.82	0.82
			0.84	0.84
131	Tyr	α	4.55	4.56
		β	3.03	2.93
		β′	3.09	3.08
		3,5-H	6.88	6.82
		2,6-H	7.18	7.12
132	Arg	α	4.25	4.28
		β	1.78	1.84
		γ	1.57	1.64
		δ	3.22	3.18
133	Lys	α	4.30	4.28
		β	1.84	1.86
		γ	1.42	1.46
		δ	1.72	1.76
		ε	2.95	3.00
134	Ala	α	4.26	4.34
		β	1.34	1.34
135	Asn	α	4.71	4.71
		β	2.76	2.75
		β′	2.85	2.85
136	Lys/Ala	α	4.30	4.34
		β	1.84	1.42
		γ	1.42	–
		δ	1.72	–
		ε	2.95	–
137	Ser*	α	4.49	4.48
		β	3.85	3.88
		β′	3.94	3.92
138	Ser*	α	4.49	4.48
		β	3.85	3.88
		β′	3.94	3.92
139	Lys	α	4.30	4.28
		β	1.84	1.86
		γ	1.42	1.46
		δ	1.72	1.76
		ε	2.95	3.00

* Assignments interchangeable.

cysteine residue in the peptide was protected with an acetamidomethyl group. Peptides were shown to be homogeneous by the production of a single peak of C18 reversed-phase h.p.l.c. and by amino acid composition analysis. High-resolution fast-atom-bombardment m.s. analysis of CP, modified CP, disrupted CP, modified–disrupted CP and rearranged CP (Table 1) gave quasi-molecular ion peaks at m/z 2141, 2048, 2082, 1991 and 2141 respectively, consistent with their structures. A doubly charged ion corresponding to $[M+2\ H]^{++}$ at m/z 1070.5, observed in the spectrum of CP, further established the high purity of this peptide. These spectra also showed substantial A-type fragmentation from the N-terminus and Z-type fragmentation from the C-terminus, in all cases consistent with the structure of the peptide being analysed (Bae, 1993).

^{1}H-n.m.r. spectra for CP and disrupted CP were recorded at 600 MHz. The initial assignments of various resonances were performed using data from the literature (Wüthrich, 1976) assuming a random-coil structure. Rigorous assignment of all protons made use of two-dimensional experiments and are given in Table 2. The chemical shifts for all the backbone amino acid protons for CP and disrupted CP closely match with one another and agree well with the expected chemical shifts of small free peptides (Wüthrich, 1976). In addition, no resonances are observed at significantly upfield or downfield positions suggesting that both CP and disrupted CP are in a random-coil conformation (Wüthrich, 1976).

The aromatic-ring protons of the tyrosine residue resonate as two doublets at δ 6.82 and 7.12 for disrupted CP and at δ 6.88 and 7.18 for CP. The aromatic-ring protons of the phenylalanine residue resonate at δ 7.25–7.40 (multiplets) for CP and disrupted CP. Two doublets at δ 0.82 and 0.84 integrating for 3 H each were tentatively assigned to the two CH_3 groups of leucine within the consensus sequence for both CP and disrupted CP. The two CH_3 groups of leucine outside the consensus sequence in both CP and disrupted CP were unresolved and resonated at δ 0.89.

Competitive binding studies using bioassay

Heparin catalyses inhibition of factor Xa via binding to ATIII at a specific pentasaccharide site within heparin. When ATIII and factor Xa are in excess, the catalytic activity of heparin can be measured conveniently by assaying residual factor Xa activity (not inhibited by ATIII–HAH complex) using a chromogenic substrate. A commercial factor Xa assay kit was used which relies on plasma as a source of certain assay components. Although plasma contains other proteins that bind heparin, these are not believed to interact at the pentasaccharide that binds ATIII. Under the assay conditions, HAH inhibited factor Xa activity with an IC_{50} of 10 nM. The mixture of ATIII and factor Xa containing 10 nM HAH was incubated in the presence of synthetic peptide before assay of residual factor Xa activity. The addition of CP gave a concentration-dependent protection of factor Xa activity which was complete at 5 μM, but considerably less protection was afforded at the same concentration of disrupted CP or rearranged CP (Figure 1). Peptides in the absence of HAH showed no effect on factor Xa activity.

Competitive binding studies using fluorescence

The interaction between heparin and ATIII can be measured by the increase in intrinsic fluorescence of ATIII originating almost entirely from its tryptophan residues (Peterson et al., 1987). Active ATIII is needed for this heparin-induced fluorescence enhancement, suggesting that the native conformation of ATIII is required (Einarsson and Andersson, 1977). When the optimum concentration of ATIII for these fluorescence studies was being

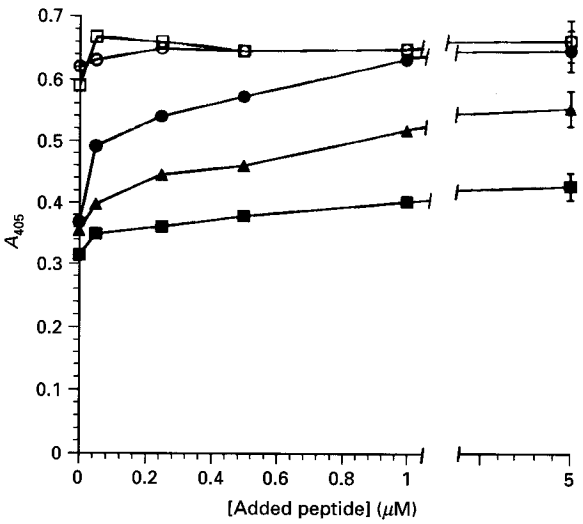

Figure 1 Competitive inhibition of heparin binding to ATIII by synthetic peptides as measured by a decrease in ATIII inhibition of factor Xa activity

Absorbance at 405 nm (a measure of residual factor Xa activity) is plotted as a function of peptide concentration. CP + HAH (●), CP (○), disrupted CP + HAH (■), disrupted CP (□) and rearranged CP + HAH (▲) are shown as solid lines.

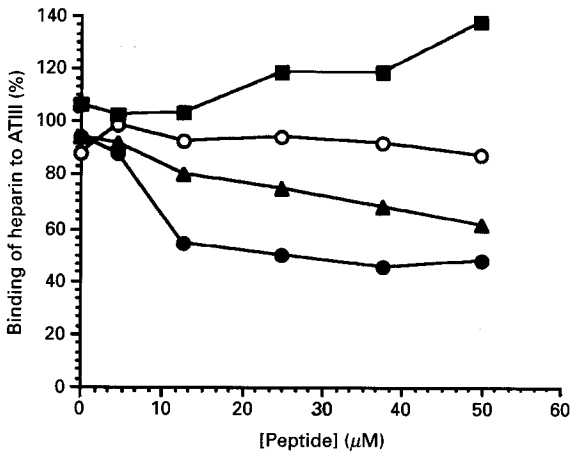

Figure 2 Competitive inhibition of heparin binding to ATIII by synthetic peptides as measured by their diminution of the fluorescence of ATIII bound to heparin

ATIII saturated with heparin (100% binding) shows maximum fluorescence, and ATIII in the absence of heparin (0% binding) shows minimum fluorescence (intrinsic fluorescence, no enhancement). Peptide was added to ATIII saturated with heparin to remove heparin from ATIII. The decrease in fluorescence provides a measure of the dissociation of ATIII from heparin. The percentage binding of heparin to ATIII is plotted as a function of the concentration of added peptide: ●, modified CP + HAH; ▲, modified CP + UFH; ■, modified–disrupted CP + UFH; ○, (modified–disrupted CP + UFH) − (modified–disrupted CP).

determined, a rapid decrease in fluorescence over time was observed. The use of a microcuvette and the addition of 500 nM BSA stabilized the fluorescence of 50 nM ATIII, suggesting that ATIII had been aggregating or adsorbing to the walls of the cuvette. Interference by the peptides also needed to be assessed before the initiation of competitive binding studies. CP and disrupted CP contain tyrosine residues and were expected to fluoresce, so these were replaced with modified CP (Y131→A) and modified–disrupted CP (Y131 and K136→A). Neither modified CP nor modified–disrupted CP showed any fluorescence at molar concentrations 2000-fold in excess of the ATIII used in these studies. Preliminary studies were undertaken to determine the molar ratio of heparin to ATIII that gave the greatest fluorescence enhancement. A 10% enhancement was obtained with HAH, UFH and LAH at molar ratios (heparin/ATIII) of 0.5, 1 and 5 respectively (results not shown). Dermatan sulphate served as a control showing no fluorescent enhancement even at concentrations in 10-fold molar excess of the heparin concentrations used. A maximum fluorescence enhancement of 50% was observed with 250 nM HAH and 50 nM ATIII and this molar ratio of 5 was used in competitive binding studies.

The addition of modified CP to ATIII–HAH disrupted this complex and reduced fluorescence enhancement in a concentration-dependent fashion (Figure 2). Surprisingly, the addition of modified–disrupted CP to ATIII–HAH complex resulted in an increased fluorescence enhancement. A control study examined the effect of modified CP and modified–disrupted CP on ATIII fluorescence in the absence of heparin and found both peptides increased ATIII fluorescence (Figure 2). Subtraction of the direct effect of modified–disrupted CP on ATIII fluorescence from the competitive effect of peptide on ATIII–HAH binding gives a true control showing no competition between modified–disrupted CP and ATIII for HAH binding. The binding constant of HAH for ATIII has been reported as 10–100 nM (Nordenman and Björk, 1978; Lindahl et al., 1984). Using these values and the observation that 25 μM modified CP is required for 50% dissociation of 50 nM ATIII from HAH, the

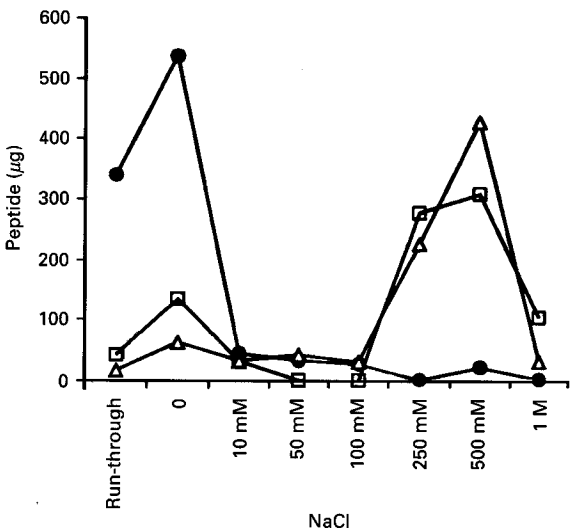

Figure 3 Elution profiles of peptides from heparin–acrylamide affinity column

CP (●), disrupted CP (□) and rearranged CP (△) are shown eluted from the column at various concentrations of salt.

affinity of modified CP–HAH binding is approx. 1000-fold less than ATIII–HAH binding, having a binding constant of approx. 3 μM. Complete reversal of ATIII binding was not achieved even with concentrations of CP of up to 50 μM, possibly because of the increased fluorescence observed when either modified CP or modified–disrupted CP are added to ATIII. At high concentrations, positively charged peptides interact with anionic ATIII, exposing its tryptophan residues. This interference increases

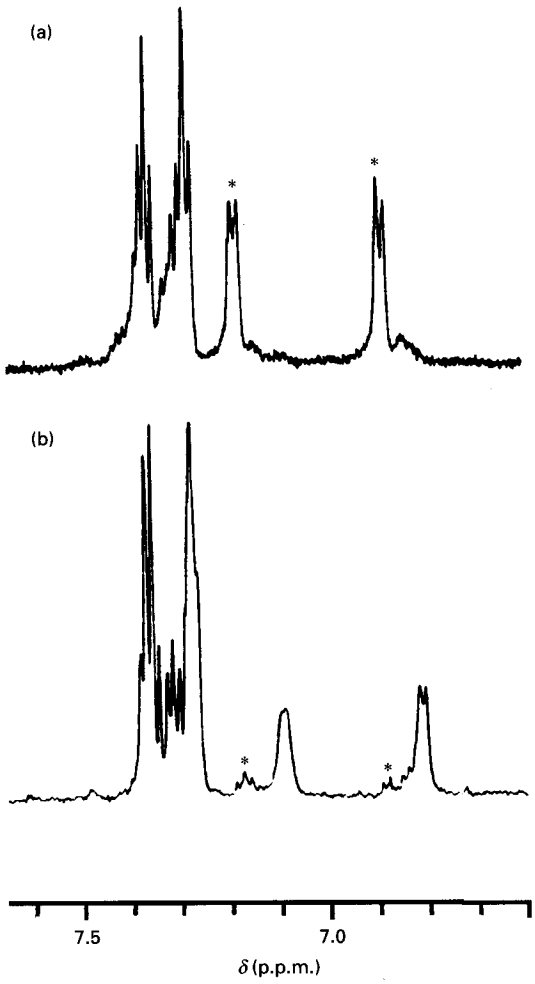

Figure 4 ^{1}H-n.m.r. studies at 600 MHz on equimolar peptide–HAH complexes

(**a**) and (**b**) are partial ^{1}H-n.m.r. spectra of the aromatic region of the 1:1 complex of heparin with CP and disrupted CP respectively. The signals marked with asterisks in (**a**) have nearly identical shift with the signals marked with asterisks in (**b**).

fluorescence, negating the diminution of fluorescence resulting from the competition of modified CP with ATIII for heparin binding.

Measurement of heparin–peptide interaction by affinity chromatography

The binding of CP, disrupted CP and rearranged CP to heparin was directly measured by affinity chromatography. Each peptide contained a single tyrosine residue which facilitated the convenient determination of peptide concentration from absorbance at 275 nm. Each peptide was loaded on to a heparin–acrylamide affinity column in water, eluted with stepwise NaCl (10 mM–1 M) washes and the peptide concentration in the column eluate determined (Figure 3). Approx. 94% of CP was eluted immediately from the heparin–acrylamide column with 4% eluted at 0.5 M NaCl. A second experiment was performed in which 400 nmol of CP was loaded on a 10 ml column. This 10-fold increase in the column volume resulted in a 10-fold increase in tightly bound peptide (50–75% of CP eluted in the 0.5 M eluent). More than 96% of rearranged CP and 90% of disrupted

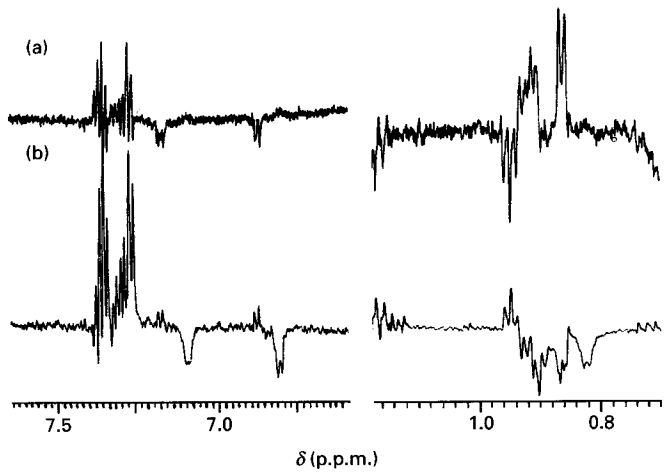

Figure 5 nOe difference spectra (600 Mz) of equimolar peptide–HAH complexes

(**a**) CP–HAH complex and (**b**) disrupted-CP–HAH complex show the effect of irradiation of the *N*-acetyl in HAH. The aromatic region (δ 7.5–6.5) of the resulting spectra shows signals corresponding to tyrosine and phenylalanine and the upfield region (δ 1.0–0.8) of the spectra shows signals corresponding to the methyl protons of leucine.

CP bound to the heparin–acrylamide column and were eluted at 0.25 and 0.5 M NaCl respectively.

Binding studies of peptides to heparin using n.m.r.

Equimolar complexes of CP–HAH and disrupted-CP–HAH were prepared in ^2H_2O, and ^{1}H-n.m.r. spectra were obtained (Figure 4). The aromatic-ring protons of the tyrosine residue of CP in the complex at δ 6.88 and 7.18 are deshielded compared with the major peaks at δ 6.82 and 7.12 corresponding to aromatic protons for disrupted CP in the complex. In addition, minor peaks were also observed at δ 6.88 and 7.18 for disrupted CP in the complex. These results suggest major differences in the electronic environment of the tyrosine residue found in the consensus sequence of CP and disrupted CP when bound to heparin. No major differences were observed in the resonances for the protons in the backbone on either peptide when complexed with heparin.

The steady-state nOe difference spectra (Figure 5) for CP–heparin and disrupted-CP–heparin complexes were obtained by low-power decoupling of protons at δ 2.1, 5.38 and 6.8. Irradiating at a frequency remote from the resonances of the complex served as a control. A positive nOe (approx. 10%) was observed at δ 0.82 and 0.84 for the CH_3 protons of the leucine residues in CP–HAH when the *N*-acetyl protons (δ 2.1) of heparin were irradiated. This also resulted in reduced signal intensity of 2,6-H and 3,5-H of the tyrosine residue in CP–HAH, whereas the multiplet corresponding to the phenylalanine protons had a net intensity of zero. These signal intensities did not change when reduced decoupler power was used to enhance sensitivity. Irradiation of the anomeric protons of HAH (δ 5.38) gave no nOe in the CP resonances. On the contrary, the peak intensities of tyrosine and phenylalanine decreased significantly. The decrease in the signal intensity of the peptide protons in the CP–HAH complex, although unexpected, is apparently a characteristic of the system. Thus, although the irradiation of CH_3 protons in the *N*-acetyl moiety of HAH resulted in a reduced signal intensity for the 2,6-H and 3,5-H resonances of the tyrosine residue in

CP–HAH (Figure 5), a net positive nOe between the *N*-acetyl moiety and aromatic protons of tyrosine for CP–HAH can be estimated at approx. 7%.

Strikingly different results were observed in nOe experiments on the disrupted-CP–HAH complex (Figure 5). Irradiation of the CH_3 protons of the *N*-acetyl moiety of HAH did not give nOe in the major signals (δ 6.82 and 7.12) assigned to the aromatic protons of 2,6-H and 3,5-H of tyrosine. However, a positive nOe (approx. 25%) was observed in the signals (δ 6.82 and 7.12) associated with a minor disrupted-CP–HAH complex. A positive nOe (approx. 25%) was also observed in the resonances assigned to the aromatic protons of phenylalanine. In contrast with the observations made for the CP–HAH complex, no nOe in the CH_3 protons of leucine in the disrupted-CP–HAH complex was observed on irradiation of CH_3 protons of the *N*-acetyl moiety of HAH. As with the CP–HAH complex, the irradiation of the anomeric protons of disrupted-CP–HAH resulted in decreased signal intensity in the 2,6-H and 3,5-H of tyrosine and phenylalanine.

DISCUSSION

Heparin contains a specific pentasaccharide sequence which occurs infrequently (approximately once in every three chains) and is responsible for the binding and activation of ATIII as a serine protease inhibitor (Linhardt and Loganathan, 1990). Affinity fractionation of heparin can be used to prepare HAH and LAH fractions, enriched and deficient respectively in the ATIII-binding site (Denton et al., 1981).

Chemical-modification reactions and site-directed mutagenesis experiments suggest several regions in the ATIII sequence responsible for heparin binding. Chemical-modification experiments suggest the binding sites to be present in the region 105–150 containing four lysine residues at 107, 114, 125 and 136 which are responsible for heparin binding (Liu and Chung, 1987; Chung, 1989). Proteolytic fragmentation of ATIII showed that a peptide containing the region 114–156 has high affinity for heparin (Smith and Knauer, 1987). Arg-129, Arg-145 and Arg-117 may also be involved in binding (Sun and Chung, 1990). Smith et al. (1990) have demonstrated that a synthetic peptide having the sequence 124–145 blocks heparin binding and activation of ATIII, thereby reducing thrombin inhibition. Finally, reduction of disulphides in ATIII indicated that the Cys-128–Cys-8 cross-link is important for heparin binding and its chemical reduction is greatly reduced in the presence of heparin (Sun and Chung, 1989). All of this evidence suggests that the heparin-binding site in ATIII is situated in the region 105–150 which contains a cluster of basic residues which are responsible for the binding.

In 1989 Cardin and Weintraub proposed a general theory for proteins that bind to heparin or other acidic polysaccharides. These heparin-binding proteins contain a specific sequence of basic and non-basic amino acid residues called a 'consensus sequence'. They proposed that the binding sites of proteins contain a cluster of high positive-charge density (basic residues) and that these basic amino acid residues are in a special arrangement that promotes electrostatic interaction with the acidic groups of glycosaminoglycans such as heparin. One feature of this consensus sequence in heparin-binding proteins is that it is composed of both basic and hydropathic residues. Computer modelling of these specific sequences was based on experimentally defined heparin-binding domains in various heparin-binding proteins. The regions shown are characterized by two 'consensus sequences' (XBBXBX) and (XBBBXXBX), where B has a high probability of occurrence of a basic amino acid residue and X is a hydropathic residue. Thus the consensus sequence represents clusters of two or three basic residues separated by two or three hydropathic residues, terminated by one or more basic residues. Helical wheel diagrams (Cardin and Weintraub, 1989) of peptides containing consensus sequences segregate the basic residues to one face, forming a cluster of high positive-charge density at which heparin may bind.

ATIII contains a consensus sequence of the (XBBBXXBX) type corresponding to amino acid residues 130–137 (LYRKANKS). This ATIII sequence is not a perfect match with the consensus motif (Y131 is not a basic residue), and thus it might be expected to show weaker heparin binding than sequences found in proteins such as the fibroblast growth factors (Volkin et al., 1993). To determine whether this consensus sequence in ATIII is the true heparin-binding site, we synthesized five peptides with amino acid sequences closely corresponding to sequence 123–139 of human ATIII. The first peptide had an amino acid sequence identical with sequence 123–139 of ATIII and was called the consensus peptide (CP); in the second, disrupted CP, one of the basic amino acids (K136), which according to the CP theory is always basic, was replaced by A. In a third, modified CP, Y131 of the consensus peptide was modified by a conservative substitution with A. The fourth, modified–disrupted CP, contained two substitutions (K136→A and Y131→A). Finally, in the fifth, rearranged CP, the amino acid sequence of the CP was totally rearranged.

A factor Xa assay was used to examine competitive heparin binding to ATIII and the peptides. These studies clearly demonstrated that CP could compete with ATIII for binding to HAH, whereas disrupted CP and rearranged CP could not (Figure 1). Similar results were obtained using fluorescence spectroscopy to measure peptide binding to HAH. Not only could competitive binding of modified CP be demonstrated (Figure 2) but a binding constant of 3 μM could be estimated using this method. No competition for heparin binding was observed between modified–disrupted CP and ATIII.

Direct binding of CP, disrupted CP and rearranged CP to heparin was studied using heparin-affinity chromatography. The columns were prepared with UFH and thus had a low ratio (approx. 1:20) of ATIII pentasaccharide binding sites to total pentasaccharide sequences. Only a small fraction of CP bound to the heparin-affinity matrix, suggesting that CP was selectively binding to the few ATIII pentasaccharide binding sites. Disrupted CP and rearranged CP bound to immobilized heparin at virtually all the available pentasaccharide sites. These results suggest a lack of specificity of peptide binding in the absence of the consensus sequence. The affinity of CP for immobilized heparin was approx. the same or slightly higher than the affinity of disrupted CP and rearranged CP for immobilized heparin.

The possible modes of binding of CP and disrupted CP to HAH are shown in Figure 6. The ^{1}H-n.m.r. spectra (Figure 4) of the equimolar complex CP–HAH and disrupted-CP–HAH show broadening of peptide resonances, especially those of the aromatic protons of tyrosine, suggesting that binding occurs for both peptides. These data rule out model (a) in Figure 6. The controlled stoichiometry of the complexes and weak binding for both peptides suggests that a 1:1 complex is formed, which rules out model (d) in Figure 6.

The aromatic-ring protons of the tyrosine residue in the equimolar complexes resonate at distinctive positions (Figure 4). This provides the most important clue in elucidating the differences between CP–HAH and disrupted-CP–HAH complexes. The CP–HAH complex shows only a single set of 2,6-H and 3,5-H resonances, ascribable to a single microenvironment for the tyrosine residue. In contrast, the disrupted-CP–HAH complex

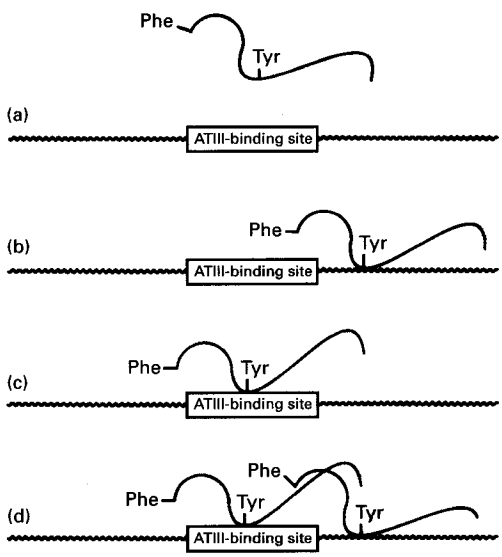

Figure 6 Model for peptide binding to heparin

The straight line represents heparin and the box in this line represents the ATIII pentasaccharide binding site in heparin. The curved lines represent peptide. Four models are shown: (**a**) no binding between peptide and heparin; (**b**) binding between peptide and heparin outside the ATIII-binding site; (**c**) binding between peptide and heparin at the ATIII-binding site; and (**d**) multiple binding of peptide to heparin both within and outside the ATIII-binding site.

shows two sets of resonances for the aromatic protons of the tyrosine residue in the ratio 93:7. The chemical shifts for the minor set of tyrosine resonances for the disrupted-CP–HAH complex correlate well with the chemical shifts of the tyrosine resonances for the CP–HAH complex, suggesting a similar microenvironment. The major set of aromatic protons of the tyrosine residue in the disrupted-CP–HAH complex resonate at a distinctive shielded position, suggesting that the major microenvironment of tyrosine in the disrupted-CP–HAH complex is different from the microenvironment of the tyrosine residue in the CP–HAH complex. Thus the ^{1}H-n.m.r. data suggest that these peptide–HAH complexes are the result of different interactions corresponding to models (b) and (c) in Figure 6.

The electrostatic interaction between cationic peptide and anionic heparin results in the two chains being more than 0.5 nm apart. At these distances a transient nOe, giving a better estimation of distance, becomes too weak to observe. A steady-state nOe is not as limited, and hence provides a better alternative for studying interaction. The steady-state nOe difference experiments on CP–HAH and disrupted-CP–HAH complexes yield distinctly different results (Figure 5). The structure of the ATIII-binding site in porcine mucosal heparin is well established and contains a single *N*-acetylglucosamine residue (Loganathan et al., 1990). Only 30–40 % of the chains in porcine mucosal heparin contain a pentasaccharide binding site (Linhardt et al., 1992a). Disaccharide compositional analysis (Linhardt et al., 1992b) and 600 MHz proton n.m.r. analysis demonstrate less than one *N*-acetylglucosamine residue in each heparin chain. Thus approx. 40–50 % of the *N*-acetylglucosamine residues are within the ATIII pentasaccharide binding site. Although the 3-*O*-sulphated glucosamine residue might represent a more specific marker for this site, its resonances were insufficiently resolved for nOe experiments. For this reason, the CH_3 protons of the *N*-acetylglucosamine residue were irradiated. A positive nOe was observed for CH_3 of the leucine residue (because of their nearly equivalent chemical shifts it was not possible to distinguish between Leu-126 and Leu-130) on irradiation of the CH_3 protons of the *N*-acetyl moiety of heparin in the CP–HAH complex. This nOe suggests close spatial proximity between the *N*-acetyl group of heparin (found primarily in the ATIII pentasaccharide binding site of porcine mucosal heparin) and a leucine residue (possibly Leu-130) within the consensus sequence. This binding of CP to the ATIII pentasaccharide binding site in HAH supports model (c) in Figure 6. The positive nOe observed for the aromatic-ring protons of phenylalanine (major resonances) on irradiation of the *N*-acetyl protons of heparin in the disrupted-CP–HAH complex suggests close contact between the phenylalanine residue of the peptide and the *N*-acetyl group in heparin. This supports disrupted-CP binding to HAH at a site different from the ATIII pentasaccharide binding site as depicted in model (b) (Figure 6). The observation of significant positive nOe between the *N*-acetyl moiety in heparin and the minor tyrosine resonances of the disrupted CP suggests the presence of a minor binding mode where disrupted CP can also occupy the ATIII pentasaccharide binding site [model (c) in Figure 6]. Although a positive nOe was not directly observed for the tyrosine aromatic protons of the CP–HAH complex on irradiation of the *N*-acetyl group, control experiments showed that the signal intensities of the tyrosine resonances in both the CP–HAH and disrupted-CP–HAH complexes decreased on irradiation, irrespective of the frequency and decoupling power. These observations suggest that the reduced intensity of the negative signal, observed for tyrosine in CP–HAH on *N*-acetyl irradiation, is actually the result of a positive nOe. This observation also supports the close spatial proximity between tyrosine in CP and the *N*-acetyl group in HAH, consistent with model (c) in Figure 6.

In conclusion, this study demonstrates that peptides with ATIII consensus sequences and those with disrupted consensus sequences both bind to heparin. Competitive-binding studies and direct-binding studies using affinity chromatography suggest that CP binds specifically to the ATIII pentasaccharide binding site, whereas disrupted CP binds non-specifically to all pentasaccharide sequences within heparin. Finally, ^{1}H-n.m.r and nOe studies confirm the binding specificity of CP and suggest a model for both specific and non-specific peptide–heparin interactions. From modelling studies, Grootenhuis and van Boeckel (1991) suggested that basic residues 125, 129, 132 and 136, all found within our synthetic CP, contribute to heparin bindings. Interestingly, basic residues 125 and 129 are outside the Cardin and Weintraub (1989) consensus sequence (130–137). Our data are consistent with the presence of both LYRKANKS (130–137) and LNCRLY (125–131) binding sites or a larger binding site extending from 125–136. The exact alignment of the consensus sequence and pentasaccharide binding site is still unclear. Quantitative nOe measurements and molecular-modelling experiments may be required to deduce the exact structure of the bound complex.

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