Mapping the heparin-binding domain of boar spermadhesins

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Abstract Boar spermadhesins are a group of seminal plasma, heparin-binding proteins which appear to be involved in sperm capacitation and gamete interaction. Using a proteolytic protection assay we have identified regions of AQN-1, AQN-3, PSP-I and AWN which remain attached to a heparin-Sepharose column following in-column digestion of bound spermadhesins with chymotrypsin and elastase. In addition, the complete amino acid sequence of spermadhesin AWN was synthesized as overlapping peptides, and their ability to bind to a heparin-Sepharose column and to inhibit the interaction of soluble heparin with purified ELISA plate-coated AWN was tested. Both approaches gave similar results and as a whole showed that different regions of AWN may converge in its tertiary structure to form a composite heparin-binding site. The conformational heparin-binding surface resides on the GFCC′C″ face of the proposed structural model for AWN and is in an opposite location to the carbohydrate-binding region of the spermadhesin.

Key words: Spermadhesins (boar); AWN-1; CUB domain; Heparin-binding mapping; Proteolytic protection assay; Synthetic peptide

1. Introduction

Heparins are linear strongly acidic glucosaminoglycans composed of repeating units of D-glucosamine and hexuronic acid residues substituted with *O*-sulphates and *N*-sulphonates produced by most animal cells [1]. Heparin and heparan sulphate glycosaminoglycans (GAGs) bind to a wide variety of physiologically and developmentally relevant cell surface-associated, secreted, and extracellular matrix proteins, and this interaction appears to have important roles in diverse biological processes [2]. Heparin and heparan sulphate GAGs exert their effects by modulating the activity of proteins to which they bind. The list of proteins with GAG-binding properties implicated in their biological roles is steadily increasing, and includes enzymes, enzyme inhibitors, extracellular matrix proteins, viral coat glycoproteins, blood coagulation factors, chemokines, growth factors, lectins, cell adhesion molecules, etc. [2].

Heparin-like GAGs secreted by the epithelium of the female reproductive tract, particularly at high concentration during the follicular phase of the estrous cycle, have been shown to invoke sperm capacitation in a number of mammalian species [3], and the effects of GAGs appear to be mediated by seminal plasma heparin-binding proteins which coat the sperm surface at ejaculation [4].

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The major heparin-binding proteins (HBPs) of several domestic mammalian species have been thoroughly investigated. The HBPs of bull and stallion seminal plasma, termed PDC-109, BSP-A3, and BSP-30K, and HSP-1 and HSP-2, respectively, are polypeptides with 109–121 amino acid residues made up of one or more 13–15-residue *O*-glycosylated N-terminal repeats followed by two tandemly arranged domains each sharing the consensus sequence of fibronectin type-II modules (see [5] and references therein). The heparin-binding proteins of boar seminal plasma belong to another protein family, called spermadhesins [6], whose members are structurally unrelated to the bovine and equine HBPs. Boar spermadhesins (AQN-1, AQN-3, PSP-I and AWN) are a group of 14–16 kDa seminal plasma and sperm-associated lectins [7], which are thought to play a major role in sperm capacitation [8] and gamete interaction [9].

The species-specific modulation exert by different heparin-binding proteins on sperm physiology may depend on how they interact simultaneously with GAGs ligands and sperm surface moieties. Bovine seminal plasma heparin-binding proteins have been shown to possess affinity for phosphorylcholine, suggesting a manner by which they become attached to the sperm surface [10]. Boar spermadhesins, depending on their aggregation state, may bind to the sperm surface via phosphorylethanolamine-containing lipids or become coated as an aggregated coating material [11]. The aim of this study was the characterization of molecular determinants of boar spermadhesins for binding heparin.

2. Materials and methods

Freshly ejaculated boar sperm was collected with an artificial vagina. Spermatozoa and seminal plasma were separated by centrifugation at 12,000 × *g* for 15 min. 100 ml seminal plasma were dialyzed overnight against 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA 0.025% sodium azide, pH 7.4 (TBS), and fractionated by affinity chromatography on a heparin-Sepharose CL-6B column (16 × 3 cm) (Pharmacia) equilibrated in the same buffer. The column was washed until the absorbance of the eluate at 280 nm reached baseline level. For proteolysis of the bound material, the column was recycled overnight at 23°C with 0.1 mg/ml of either porcine pancreatic α-chymotrypsin (EC 3.4.21.1) or elastase (3.4.21.36) (Sigma) in TBS. After washing, bound peptides were recovered eluting with TBS containing 1 M NaCl, and separated by reversed-phase HPLC on a Lichrospher RP100 C18 column (0.4 × 25 cm, 5 μm particle size) (Merck) eluted at 1 ml/min with a gradient of 0.1% (v/v) trifluoroacetic acid in water (solution A) and acetonitrile (solution B) as follows: isocratically with 5% solution B for 5 min, followed by 5% to 25% solution B for 80 min, and 25% to 70% solution B for 30 min.

Peptides were characterized by N-terminal amino acid sequence analysis using an Applied Biosystems 477A/120A sequencer, and amino acid analysis using an Alpha Plus (Pharmacia) amino acid analyzer

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after sample hydrolysis with 6 M HCl for 24 h at 110°C in evacuated and sealed ampules. Peptide sequences were aligned in the amino acid sequences of AWN [12], PSP-I [13,14], AQN-1 [15], AQN-3 [16], and seminal plasma trypsin inhibitor [17].

The following overlapping peptides, which cover the complete amino acid sequence of porcine spermadhesin AWN (133 residues) [12], were synthesized using a standard Fmoc-protocol [18], using preloaded *p*-alkoxybenzyl alcohol resins or a Rink-amide resin (peptide P10), on a SMPS 350 automated multiple peptide synthesizer (Zinsser, Frankfurt) equipped with a 48-syringe synthesis block (Multisyntech, Bochum): P1, ^{1}AWNRRSRSAGGVLRD^{15}; P2, ^{13}LRDPPGKIFNSDGPQ^{27}; P3, ^{25}GPQKDAVWTIKVKPHF^{40}; P4, ^{37}KPHFHVVLAIPPLNL^{51}; P5, ^{46}AIPPLNLSAGKEYVE^{59}; P6, ^{53}AGKEYVELLDGLDG^{63}; P7, ^{61}LDGPPGSEIIGKIAG^{75}; P8, ^{73}IAGGISLVFRSSSNI^{87}; P9, ^{81}FRSSSNIATIKRLRT^{95}; P10, ^{89}TIKRLRTSGHRASPF^{103}; P11, ^{97}GHRASPFHIYYYADP^{111}; P12, ^{109}ADPEGPLPFPYFERQ^{123}; and P13, ^{119}YFERQTIIATEKNIP^{133}. The four cysteine residues of AWN (positions 9, 30, 53, and 74), which form two disulphide bridges between nearest neighbor residues [12] were substituted by alanine.

For testing the ability of synthetic peptides P1–P12 to bind heparin, the peptides (1 mg/ml in TBS) were chromatographed on a heparin-Sepharose CL-6B column (1 × 10 cm) using an FPLC equipment (Pharmacia) and the same chromatographic conditions as above. The non-bound and bound fractions were characterized by reversed-phase HPLC on a Lichrospher RP100 C18 column (0.4 × 25 cm, 5 μm particle size) (Merck) eluted at 1 ml/min with a gradient of 0.1% (v/v) trifluoroacetic acid in water (solution A) and acetonitrile (solution B) isocratically with 5% solution B for 5 min, followed by 5% to 30% solution B for 50 min, and 30% to 70% solution B for 20 min. Isolated peptides were identified by amino acid analysis.

The ability of synthetic peptides P1–P10 to inhibit the binding of biotinylated heparin to purified AWN-1 was assessed using an enzyme-linked immunosorbent assay (ELISA). To this end, 1 μg of AWN (purified as described in ref. [6]) in 100 μl 50 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.5, were coated overnight at 4°C onto ELISA plate wells. After blocking with TBS buffer containing 5% (w/v) bovine serum albumin (BSA) for 2 h at 37°C, the immobilized proteins were incubated with 100 μl of solutions containing 60 ng biotinylated heparin preincubated for 1 h at 37°C with increasing concentrations (0.1–100 nmoles) of a given synthetic peptide in TBS. After 3×washing with TBS containing 0.5% (w/v) BSA, samples were incubated with 100 μl streptavidin–peroxidase (1:4000, v/v) in washing buffer, washed 3 times, and developed with 100 μl/well of 2 mg/ml *o*-phenylendiamine in 20 mM citric acid, 50 mM NaH_2PO_4 buffer, pH 5, containing 70 μl H_2O_2 (30%)/100 ml. The color developed was measured at 492 nm with an automated micro-ELISA reader (ICN).

3. RESULTS

3.1. Location of heparin-binding regions using a proteolytic protection approach

As a first approach for characterizing heparin-binding regions of boar spermadhesins, seminal plasma was fractionated by affinity chromatography on heparin-Sepharose and the bound proteins (a mixture of AQN-1, AQN-3, PSP-I, and AWN [6,14]) were proteolytic degraded with either α-chymotrypsin or elastase. The rationale behind this approach was that those protein regions interacting with heparin should be protected from proteolysis and would remain bound to the affinity matrix.

Table 1 shows the results obtained and Fig. 1 the location of the chymotryptic (C-) and elastase-derived (E-) heparin-bound fragments within the amino acid sequences of boar spermadhesins. Degradation with chymotrypsin yielded 11 fragments, which mapped to three different regions of the parent proteins. Fragments C-3, C-10, C-1, and C-7 were derived from the N-terminal region of AQN-1, PSP-I, AQN-3, and AWN, respectively. Fragments C-10 and C-7 contained each two disulphide-bonded peptides, whereas C-3 and C-1 corresponded to the loop region formed by the conserved disulphide bridge between the first two cysteines of the proteins [12,13,15,16]. Fragments C-4, C-8, and C-11, contained each two peptides disulphide-bonded by the second conserved cystine of spermadhesins AQN-1 [15], PSP-I [13], and AWN [12]. Peptide C-5 corresponded to a region of AQN-3 which aligns with the C-terminal part of peptides contained in C-4, C-8, and C-11 (Fig. 1). Finally, fraction C-2 contained two peptides encompassing a highly homologous, tyrosine-rich region at the C-terminal part of AQN-1 and AWN.

When heparin-bound seminal plasma proteins were degraded with elastase, only one peptide corresponding to a spermadhesin molecule (E-1, AWN 101–109) (Table 1) was found. This fragment contained the sequence found in C-2 flanked by several other residues at the N- and C-termini (Fig. 1). Other heparin-bound fragments corresponded to polypeptide stretches of a seminal plasma acrosin inhibitor [17] (Table 1).

3.2. Analysis of the heparin-binding capability and AWN-heparin-binding inhibitory activity of synthetic peptides

To narrow down the heparin-binding domain of AWN, the complete amino acid sequence of the protein was synthesized as 11–15-mer overlapping peptides. When these peptides were probed for their capability to bind to heparin-Sepharose (either individually or as a mixture) it was found that peptides P2, P5, P6, P7, P12, and P13 eluted with the flow-through fraction; peptides P3, P4, and P11 were retarded in the column and eluted under isocratic conditions with 4–5 column volumes, and peptides P1, P8, P9, and P10 bound to the affinity matrix and were eluted with 1 M NaCl. Alignment of these peptides with the AWN structure is shown in Fig. 1.

The ability of synthetic peptides to interfere with the binding of biotinylated heparin to purified AWN was tested using competitive ELISA. Only peptides P8, P9, P10, and P11 inhibited AWN-heparin interaction in a concentration-dependent man-

Table 1
Characterization of proteolytic products which remained bound to heparin-Sepharose after digestion of heparin-bound boar seminal plasma proteins with α-chymotrypsin (C-) or elastase (E-)

Peptide	N-terminal amino acid sequence	Corresponds to	
C-1	NYSGWISY	AQN-3	15–22
C-2	DIYYY	AQN-1	106–108
	HIYYY	AWN	106–108
C-3	RISTYEGPKTD	AQN-1	19–29
C-4	ACGKEY	AQN-1	52–57
	NYGKLCSGIGL	AQN-1	69–79
C-5	TVYQSS	AQN-3	79–84
C-6	TYQSSSNAL	AQN-1	80–88
C-7	AWNRRSRSCGGVL	AWN	1–13
	NSDGPQKDCVW	AWN	22–32
C-8	NLTCGKEY	PSP-I	47–54
	CEGLSILNRGSSGM	PSP-I	71–79
C-9	RDLSGRISTYEGPKT	AQN-1	14–28
C-10	HACGGRL	PSP-I	4–10
	TECVW	PSP-I	25–29
C-11	SCGKEY	AWN	52–57
	CGGISLVFRSSSNI	AWN	74–87
E-1	SPFHIYYYA	AWN	101–109
E-2	KKTRKEPDXD	AI	4–17
	HLFFXTREM	AI	18–69
E-3	ARSKKTRKEP	AI	1–69

Peptides were aligned in the amino acid sequences of AWN [12], PSP-I [13,14], AQN-1 [15], AQN-3 [16], and acrosin inhibitor (AI) [17].

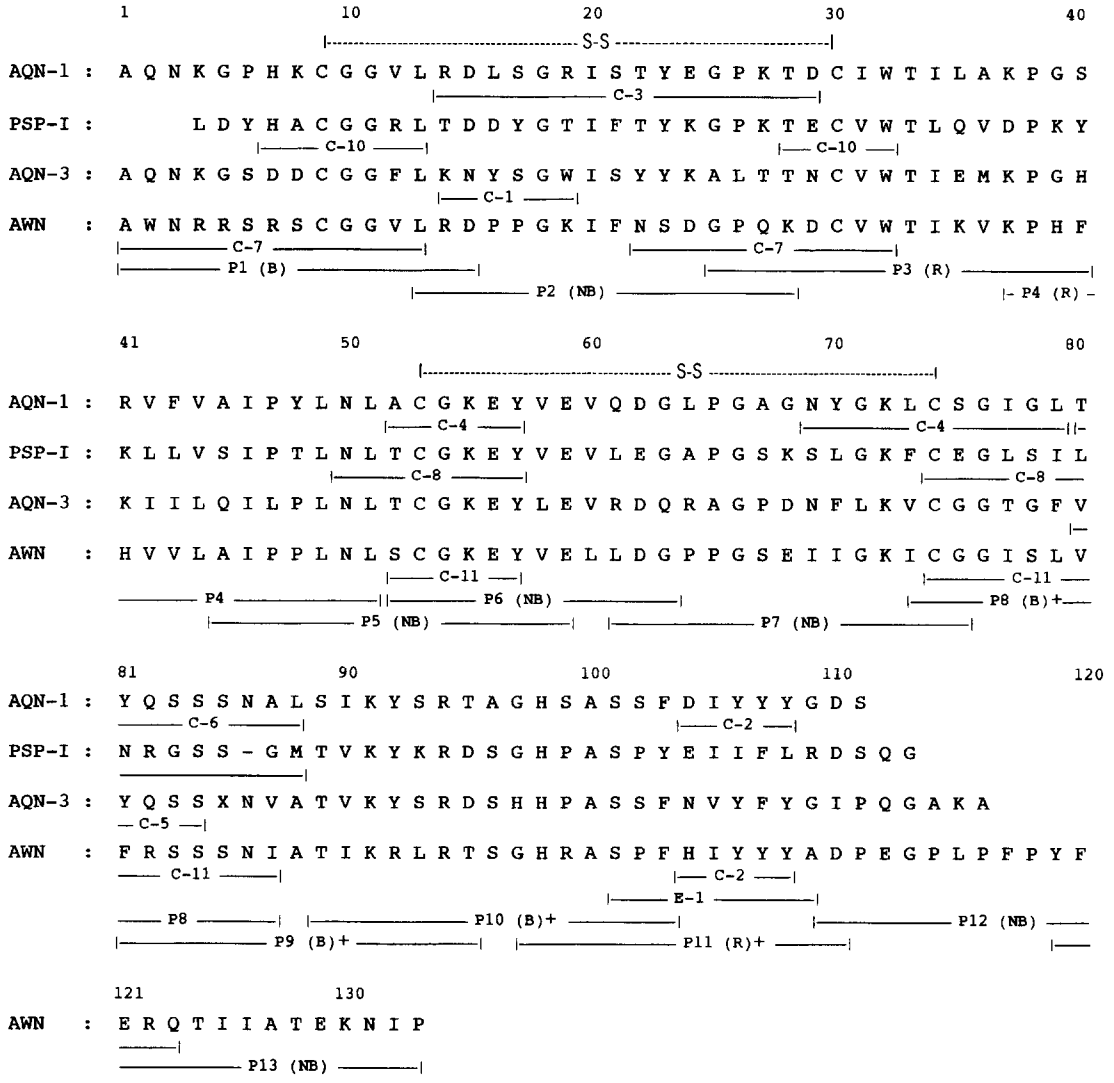

Fig. 1. Alignment of the amino acid sequences of boar spermadhesins AQN-1 [15], PSP-I [13,14], AQN-3 [16], and AWN [12] showing the chymotryptic (C-) and elastase-derived (E-) fragments obtained by in situ proteolysis of heparin-bound seminal plasma proteins. The location of overlapping synthetic peptides P1–P13 of AWN employed to determine heparin-binding capability and AWN-heparin-binding inhibitory activity are shown beneath the AWN sequence. NB, R, and B, denote those peptides which were nonbound, retarded, or bound in the heparin-Sepharose column. +, peptides with AWN-heparin-inhibitory activity.

ner (Fig. 2) with IC_{50} values of 2.5, 1.4, 5.5, and 6.0 μM, respectively.

4. Discussion

The results on in situ-degradation of heparin-Sepharose-bound boar seminal plasma proteins summarized in Table 1 and Fig. 1 indicate that discrete regions of spermadhesins AQN-1, PSP-I, AQN-3, and AWN may converge, as a result of the tertiary structure of the proteins, to form a composite heparin-binding domain. Except for C-2 (and E-1) which are tyrosine-rich peptides, all other heparin-bound fragments contain one or more basic residues (Fig. 1). This is in agreement with recent studies showing that glycosaminoglycan-binding sites are typically extended clusters of positively charged amino acids distributed along the surface of a protein, or in a shallow groove [19] termed a 'cationic cradle' [20]. In addition, aromatic residues have been shown to contribute to the protein-heparin binding affinity [21]. It is therefore conceivable that tyrosine interacts with heparin by formation of hydrogen bonds between its hydroxyl group and the secondary alcoholic groups of the heparin carbohydrate units, and through a stacking interaction between the aromatic ring of tyrosine and the carbohydrate moieties of heparin. Such interaction has been observed in crystal structures of galactose-binding lectins [22]. However, there is no example of a GAG-protein complex determined by X-ray crystallography or NMR methods and current concepts about protein-GAG interactions have been derived by computer-assisted modelling studies (reviewed in [2,19]).

The heparin-bound fragments were largely derived from topologically equivalent stretches within the amino acid sequences of AQN-1, PSP-I, AQN-3, and AWN comprising regions around the two conserved disulphide bridges and at the C-terminal part of the molecules (Fig. 1). However, differences

were also observed. Hence, while AQN-1 and AQN-3 contained heparin-bound peptides mapping to the loop region between the first two cysteines (C-3 and C-1, respectively) (Table 1, Fig. 1), PSP-I and AWN heparin-bound fragments C-10 and C-7, respectively, each contained two disulphide-bonded peptides. The amino acid sequences AQN-1 ^{17}SGRISTY^{23} and AQN-3 ^{14}KNYSGWISY^{22} of heparin-bound peptides C-3 and C-1, respectively, display similarity with the sequence stretches ^{99}RGRISCT^{105} and ^{189}SGRITCT^{195} involved in the binding of heparin to fibronectin [23]. Furthermore, similarly to AQN-1 and PSP-I, the fibronectin sequences are located in disulphide-bonded loops [24]. On the other hand, the amino acid sequence of AWN ^{4}RRSRS^{8} contained in peptide C-7 (Table 1, Fig. 1) resembles the consensus sequence XBBXBX (B, basic amino acid) found in the heparin-binding regions of many diverse proteins [25]. This evidence suggests that the topology of the heparin-binding domain may not be absolutely conserved in different spermadhesins.

AWN is the most thoroughly investigated spermadhesin molecule regarding its ligand-binding capabilities and relative location of active sites (reviewed in [7]). We choose therefore AWN to narrow down the architecture of its heparin-binding domain(s). To this end, the heparin-binding capability and AWN-heparin-binding inhibitory activity of synthetic peptides (P1–P13, Fig. 1), which cover the complete amino acid sequence of AWN, were investigated. Peptides of both the N- and the C-terminal regions of AWN retained heparin-binding activity. Furthermore, peptides P8, P9, P10, and P11 (encompassing residues 73–110) competed with AWN for binding to heparin (Fig. 2). These region of AWN which includes the heparin-bound proteolytic fragments C-11, C-2, and E-1 (Table 1, Fig. 1), may therefore contain essential residues for heparin-binding. The region 73–110 spans β-strands F and G and the loops connecting β-strands E-F and F-G in the proposed structural model for AWN [26,27] shown in Fig. 3, and, therefore, would be located on the same face of the molecule. On the other hand, the fact that peptides P1, P3 and P4 bound to heparin-Sepharose or were retarded by the affinity matrix, but did not interfere with AWN-heparin interaction, might suggest that AWN

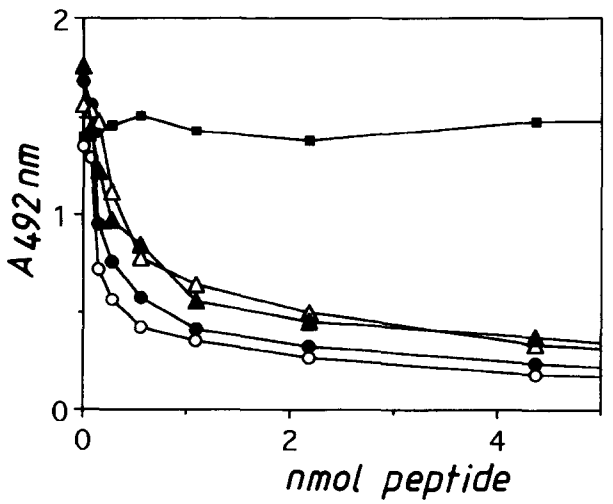

Fig. 2. AWN-heparin-inhibitory activity of synthetic peptides derived from the AWN amino acid sequence. ■, P1, P2, P3, P4, P5, P6, P7, P12, P13; ▲, P11; ●, P9; ○, P8; △, P10.

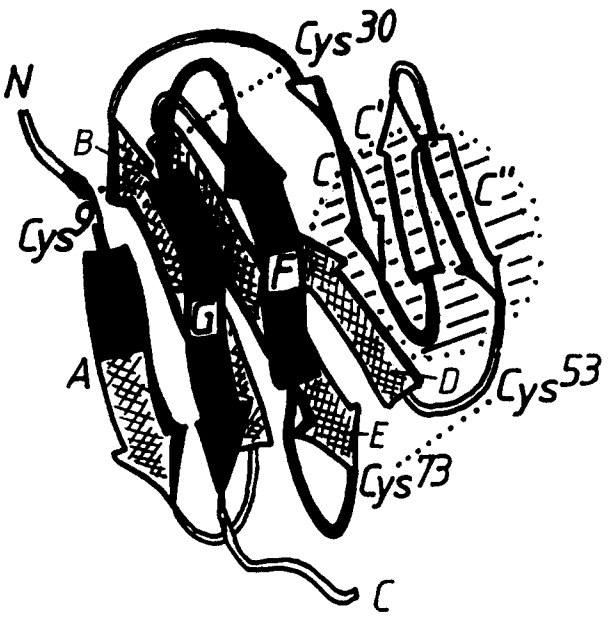

Fig. 3. Proposed structural model of AWN [25,26] schematically depicting the location of the primary (on strands F and G and loop regions between strands E–F and F–G) and secondary (polypeptide stretch 4–8 and N-terminal part of strand A) heparin-binding regions (black filled regions) characterized in this study. The proposed topological relation of the composite heparin-binding domain to the carbohydrate-binding region (stripped circle) on the GFCC'C'' face of the molecule is highlighted. N and C, N- and C-termini, respectively. The position of disulphide bridges between cysteine residues (Cys) 9–30 and 53–73 [12] is shown.

possess an additional, secondary heparin-binding region. This region would comprise the polypeptide chain 4–8, the N-terminal part of β-strand A, and the loop between β-strands C and C' (Fig. 3). The loop connecting strands C-C' would lay on the GFCC'C'' face of the AWN model while the N-terminal stretch might not be constraint in a fixed conformation. It is noteworthy that similar to our results concerning AWN ^{4}RRSR^{8}, the RRAR sequence, when present in a synthetic peptide, has been shown to have weak affinity for heparin [28,29]. Furthermore, this same RRAR sequence exists in the carboxylterminal lobe of lactoferrin where it is not functional as a GAG-binding site [30]. These results together with recent mutagenesis studies on fibronectin module III-13 have point out that the BBXB consensus sequence by itself has little affinity for heparin even when presented in the context of a folded domain [31].

As a whole, the results obtained by proteolytic protection assay and synthetic peptide approaches are consistent and complement each other. Noteworthy, the heparin-binding domain of AWN appears to be located in the same face where the carbohydrate-binding surface has been mapped [13,27,32] (schematically depicted in Fig. 3). If the structural model of AWN turns to be correct, the heparin-binding region would run in NW-SE direction of the GFCC'C'' face (Fig. 3) while the carbohydrate-binding region would be located on the CC'C'' corner. This may provide a structural basis for the observation that heparin inhibits the binding of carbohydrate-ligands (zona pellucida) to AWN [6] by interacting with a region different than the oligosaccharide recognition domain [27,32].

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