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Importance of Specific Amino Acids in Protein Binding Sites for Heparin and Heparan Sulfate

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Heparin and heparan sulfate bind a variety of proteins and peptides to regulate many biological activities. Past studies have examined a limited number of established heparin binding sites and have focused on basic amino acids when modeling binding site structural motifs. This study examines the prevalence of individual amino acids in peptides binding to heparin or heparan sulfate. A 7-mer random peptide library was synthesized using the 20 common amino acids. This 7-mer library was affinity separated using both heparin and heparan sulfate–Sepharose. Bound peptide populations were eluted with a salt step gradient (pH 7) and analysed for amino acid composition. Peptides released from heparin–Sepharose by 0.3 M NaCl were enriched in arginine, lysine, glycine and serine; and depleted in methionine and phenylalanine. In contrast, peptides released from heparan sulfate–Sepharose were enriched in arginine, glycine, serine, and proline (at 0.15 M NaCl). These peptides were depleted in histidine, isoleucine, methionine (not detectable) and phenylalanine. In the heparin binding sites of proteins, which have been published, the enriched amino acids were arginine, lysine and tyrosine. Depleted amino acids include aspartic acid, glutamic acid, glutamine, alanine, glycine, phenylalanine, serine, threonine and valine. This study demonstrates that heparin and heparan sulfate bind different populations of peptide sequences. The differences in amino acid composition indicate that the positive charge density and spacing requirements differ for peptides binding these two glycosaminoglycans.

Keywords: Heparin Heparan sulfate Peptide library Glycosaminoglycan–protein interactions

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INTRODUCTION

Heparin and heparan sulfate are comprised of structurally similar, linear polysaccharides that interact with a wide variety of proteins and peptides (Lindahl, 1989). Heparin is a proteoglycan produced by mast cells and basophils that is primarily composed of *O*-sulfated L-iduronic acid alternating in a 1→4 linkage with *N*- and *O*-sulfated D-glucosamine residues (Fig. 1(A)). Commercial or glycosaminoglycan heparin is a polydisperse, highly sulfated polysaccharide degradation product of the native proteoglycan and is used extensively as an anticoagulant. Heparin is the strongest acid present in the human body and the most acidic polysaccharide in nature. As a result, heparin binds to many cationic proteins giving rise to a myriad of biological activities (Lindahl, 1989). Some of these interactions have received extensive attention in recent years, including heparin's effect on angiogenesis and growth factors (Linhardt and Loganathan, 1990) and on the complement system (Edens *et al.*, 1993).

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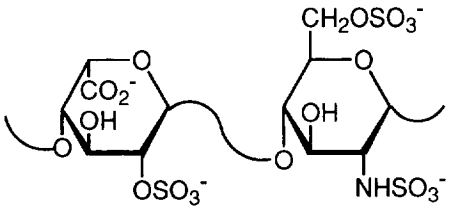
Abbreviations: *t*-Boc, *t*-Butyloxycarbonyl; *p*-MBHA, *p*-methylbenzhydrylamine; MW, molecular weight; MWCO, molecular weight cut-off.



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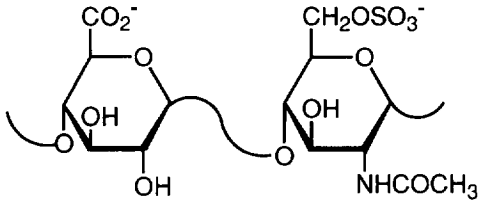

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Fig. 1. Heparin and heparan sulfate major repeating units: (A) heparin: →4) 2-deoxy-2-sulfamido-D-glucopyranosyl-6-*O*-sulfate (1→4) L-idopyranosyl uronic acid 2-*O*-sulfate (1→; (B) heparan sulfate: →4) 2-deoxy-2-acetamido-D-glucopyranosyl-6-*O*-sulfate (1→4) D-glucopyranosyl uronic acid (1→.

Heparan sulfate has a more complicated primary structure than heparin and consists of alternating 1→4 linked glucosamine (64% *N*-acetylated and 36% *N*-sulfated) and hexuronic acid (23% iduronic acid and 66% glucuronic acid) residues (Fig. 1(B)) (Toida *et al.*, 1995). Glycosaminoglycan heparin and heparan sulfate differ in the ratio of *N*-acetylation to *O*-sulfation (Gallagher and Walker, 1985). Proteoglycan heparan sulfate is found in membranes of a wide variety of cells and in the extracellular matrix. When isolated from the endothelial cell surface, heparan sulfate possesses "heparin-like" sequences, which result in its anticoagulation activity (Mertens *et al.*, 1992). Moreover, proteoglycan heparan sulfate plays an active role in cell to cell communication through the binding of growth factors, hormones and other regulators (Pettersson *et al.*, 1991; Kjellén and Lindahl, 1991).

Past studies to describe protein–glycosaminoglycan interactions have concentrated on the binding of heparin to proteins and peptides (Jackson *et al.*, 1991). Several groups have attempted to define the structural requirements in proteins and peptides that foster heparin binding (Cardin and Weintraub, 1989; Sobel *et al.*, 1992; Margalit *et al.*, 1993). Using sequence alignment, Cardin and Weintraub (1989) predicted the presence of two consensus sequence motifs with the capacity to interact with heparin. Sobel and coworkers (1992) suggested a larger consensus sequence, whereas Margalit and coworkers (1993) concluded that no consensus sequences exist for the interaction with heparin and suggested that the orientation and spacing within an α-helix or β-sheet or between adjacent strands provides the critical secondary structure for the interaction with heparin.

Most of the previous studies and theoretical modeling reports have focused on the three basic amino acids, arginine, lysine and histidine, and their spacing in the proposed heparin binding sites of a limited number of proteins. Little attention has been given to the other 17 amino acids in glycosaminoglycan recognition. Thus, more experimental data are needed to determine the primary structure requirements for proteins to interact with heparin and heparan sulfate. To obtain this information, the frequency of amino acids in proteins with experimentally defined heparin binding sites was surveyed. To examine a pool of all possible heparin or heparan sulfate binding sequences, a random peptide library was synthesized using all 20 common amino acids. The size of the library was chosen to be 7 amino acids in length in accordance with the consensus peptide theory proposed by Cardin and Weintraub (1989). The interaction of this peptide library with heparin and heparan sulfate was then examined. These studies provide an experimental basis to determine the primary structure requirements for peptides and proteins to interact with heparin and heparan sulfate.

EXPERIMENTAL PROCEDURES

Materials

t-Butyloxycarbonyl (*t*-Boc) amino acids were from Advanced ChemTech, Louisville, KY, U.S.A.; *p*-methylbenzhydrylamine (*p*-MBHA) resin (particle size 150–200 mesh, 83–115 μM, 1.7 million beads/g) was from Colorado Biotechnology Associates, Denver, CO, U.S.A.; trifluoroacetic acid was from Halocarbon Products, Augusta, SC, U.S.A.; CNBr activated Sepharose and bulk heparin–agarose resin (type II, 0.75–1 mg heparin/ml, specific activity 170 USP units/mg) were from Sigma Chemical Co., St Louis, MO, U.S.A.; heparin–Sepharose CL-6B (1 mg/ml heparin)

was from Pharmacia, Uppsala, Sweden; heparan sulfate (bovine kidney) was from Grampian Enzymes, Aberdeenshire, U.K.; Spectra/Por dialysis tubing was from Spectrum, Houston, TX, U.S.A.; Toluidine Blue O was from Fisher Scientific, Pittsburgh, PA, U.S.A. All other reagents were from either Fisher Scientific, Pittsburgh, PA, U.S.A. or Aldrich Chemical, St Louis, MO, U.S.A.

Synthesis of the random peptide library

The 7-mer peptide library was synthesized using standard *t*-Boc chemistry (Merrifield, 1963; Houghten *et al.*, 1984; Furka *et al.*, 1988, 1991) and the *t*-bag technique, in which the *p*-MBHA resin was compartmentalized in polypropylene bags (Houghten, 1985). Peptides in the library were expected to contain approximately equimolar ratios of the 20 common amino acids at each position. During each coupling cycle the bags were placed in the same reaction vessel for the deblocking and base washing steps and then separated only for the coupling reactions. Between cycles the *t*-bags were opened, and the resin rinsed into a common vessel and thoroughly mixed with very vigorous shaking. After the resin settled, the solvent was decanted, and the resin was dried. The resin was then stirred and aliquoted into 20 bags on an equal weight basis. All amino acids were blocked at the *N*-terminus with *t*-Boc. The side chains were protected as Arg (*N*-guanidino-toluenesulfonyl), Asp (*β*-cyclohexyl ester), Cys (*S*-4-methyl-benzyl), Glu (*γ*-benzyl ester), His (N_{im}-benzyloxymethyl), Lys (*N*-*ε*-2-chlorobenzyloxycarbonyl), Ser (*O*-benzyl), Thr (*O*-benzyl), and Tyr (*O*-2,6-dichlorobenzyl). After the final deblocking step, each of the 20 sublibraries (bags) was cleaved from the resin and the side chains deprotected using a standard HF/anisole procedure (Houghten *et al.*, 1986). As many as 10 intact bags of resin were cleaved simultaneously in a compartmentalized reaction vessel from Multiple Peptide Systems, San Diego, CA, U.S.A. Ethyl acetate was used to remove the residual anisole before the peptides were extracted with 15% acetic acid. After lyophilization, the 20 sublibraries were pooled on a equal weight basis. The crude peptide library was analysed for amino acid composition by the Protein Structure Laboratory, University of California, Davis, CA, U.S.A. Glu and Gln, and Asp and Asn were detected as Glx and Asx, respectively, and were present in double the concentration detected for the other amino acids, which were detected in equimolar ratios. The exceptions were Cys and Trp, which could not be quantified.

Preparation of heparan sulfate–Sepharose

CNBr activated Sepharose 6B (300 mg) was incubated with heparan sulfate (1 mg in 1 ml of 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 9.5) for 2 hr at room temperature (Danishefsky *et al.*, 1976). The mixture was then held at 4°C for 24 hr with gentle shaking. The mixture was centrifuged to recover the beads, which were washed with 16% NaCl to remove the non-specifically bound heparan sulfate (Nadkarni *et al.*, 1994). The gel was then incubated with 0.2 ml 1 M aqueous ethanolamine (pH 9.0) for 2 hr at room temperature to block residual binding sites. Excess ethanolamine was removed by washing with 16% NaCl. The gel contained 0.2 mg heparan sulfate per ml of swelled beads as determined by Toluidine Blue assay (Smith *et al.*, 1980).

Affinity fractionation of peptide library

Columns were prepared to contain heparin–agarose (50 ml), heparin–Sepharose (2 ml), or heparan sulfate–Sepharose (1 ml). Buffers, included 5 mM Na_2HPO_4, pH 7; and 0.05, 0.15, 0.3, 1 and 2 M NaCl in 5 mM Na_2HPO_4, pH 7. Before each use, the resins were washed with 8 column volumes of the sodium phosphate buffer containing 2 M NaCl before being equilibrated with buffer containing no added salt. In preliminary experiments the 7-mer random peptide library was dissolved in the 5 mM phosphate buffer containing no added salt and a 3-fold molar excess of peptide to glycosaminoglycan was applied to a 1 ml heparin–agarose column; no detectable quantities of the peptide bound to the column. Subsequently, a 20 to 50-fold molar excess of peptide was applied to the heparin (average MW = 14,000) and heparan sulfate (average MW = 20,000) columns. Peptides were then eluted with 8 column volumes each of 5 mM phosphate buffer without salt and 5 mM phosphate buffer containing 0.05, 0.15, 0.3 and 1 M NaCl, successively. These fractions were dialysed by using 500 MWCO dialysis tubing against water and were then lyophilized. A portion of each fraction was analysed for amino acid composition. A single fraction was Edman sequenced. This method resulted in amino acid carry over

from cycle to cycle. This carry over can be recognized and disregarded when a single peptide is being sequenced. In the case of multiple sequences, it cannot be distinguished from amino acids occurring at low frequency within the sample. Therefore, the sequence analysis was uninterpretable.

RESULTS

Amino acid composition of known (reported) heparin binding sites

The frequencies of amino acids in sequences reported to bind heparin (heparin binding sites) were examined to define the primary structural requirements for peptide and protein interaction with sulfated polysaccharides. The heparin binding site sequences that were examined are shown in Table 1. These were previously shown to be heparin binding sites based on: (a) the ability of an analogous heparin binding peptide (synthesized or purified fragments from proteins) to bind heparin; (b) the inability of proteins mutated at the presumed active site to bind heparin; (c) the inability of chemically modified putative heparin binding residues to bind heparin; and/or (d) the ability of heparin to protect residues from chemical modification when the heparin binding protein is bound to heparin. Reported sequences containing more than 50 amino acids were not considered because they are more likely to contain discontinuous segments. Frequencies of occurrence for the 20 common amino acids in heparin binding sites (H_f) are presented in Table 2. N_f is the natural frequency at which the amino acids appear in 207 unrelated proteins (Klapper, 1977). Consequently, a H_f/N_f ratio greater than 1 for a given amino acid indicates enrichment in heparin binding sites. As might be expected, the basic amino acids arginine and lysine are by far the most commonly found amino acids in known heparin binding sites ($H_f = 14.3$ and 15.6, respectively). These H_f values represent an enrichment in known heparin binding sites of nearly 300% for arginine and an enrichment of greater than 200% for lysine ($H_f/N_f = 3.04$ and 2.23, respectively). The third basic amino acid, histidine, appeared to change less than 10% in frequency when its frequency in heparin binding sites was compared to its natural frequency in a sample representing all proteins ($H_f/N_f = 1.09$). Three neutral amino acids, leucine, tryptophan, and tyrosine, increased in frequency by greater than 15% in heparin binding sites compared to their natural frequencies ($H_f/N_f = 1.17$, 1.21 and 1.20, respectively). Due to the low representation of tryptophan, this increase may not be as significant as a 17–20% change in leucine and tyrosine. All other amino acids are present in heparin binding sites at or below their frequencies in nature ($H_f/N_f \lesssim 1$). Aspartic acid and alanine were the least frequently occurring amino acids when compared with their natural frequency in heparin binding proteins ($H_f/N_f = 0.48$ and 0.47, respectively).

Affinity fractionation of random library using heparin

The amino acid composition of the unfractionated peptide library was consistent with the synthesis of a random peptide library. The frequency of each amino acid in the library is defined as L_f and is used to normalize the results from each of the affinity fractionated samples. The heparin–agarose and heparin–Sepharose columns produced a major alteration in amino acid composition as early as the first fraction, 0.05 M NaCl in 5 mM Na_2HPO_4, pH 7. Data for the heparin–Sepharose column are presented in Table 3. The first fractions consisting of the non-binding peptides recovered in the loading buffer contained over 97% of the loaded peptides. The amino acid composition of this fraction showed no significant change from the original library and is not presented in tabular form. The predominant effect of affinity fractionation was the enrichment of the positively charged lysine and arginine residues in the binding fractions. If there had been no selection by heparin, the amino acid composition of the fraction collected in the 0.05 M NaCl wash would have been an equimolar ratio with 33 nmol of each amino acid present. Instead, 52.90 nmol of lysine and 45.27 nmol of arginine were observed in this fraction. In addition, glycine, serine and methionine were present at 39.81, 40.00 and 13.58 nmol, respectively. For the remaining amino acids, including the basic amino acid histidine, only modest changes from equimolarity were observed. The cumulative recovery of each amino acid with a given salt concentration was calculated as the sum of recovered nmol of that amino acid in successively eluted frac-

Table 1. Published heparin binding sites

Protein	Peptide sequences	Criteria	References
bFGF	^{93}FFFERLESNNYNTYRSRKYSSWYVALKR^{120}	a	(Baird *et al.*, 1988)
	^{24}YCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGV^{68}	a	(Baird *et al.*, 1988)
Antistasin	^{93}PNGLKRDKLGCEYCECRPKRKLIPRLS^{119}	a	(Manley *et al.*, 1992)
ApoE	^{144}LRKRLLRD^{151}	a	(Cardin *et al.*, 1986)
	^{211}GERLRARM^{218}	a	(Cardin *et al.*, 1986)
LPL	^{281}RKNR^{284}	b	(Evans *et al.*, 1992)
EC-SOD	^{205}REHSERKKRRRESECKAA^{222}	b	(Sandström *et al.*, 1992)
vWF	^{565}YIGLKDRKRPSELRRIASQVKYA^{587}	a	(Sobel *et al.*, 1992)
NCAM	^{131}TWKHKGRDVILKKDVRFI^{148}	b	(Kallapur and Akeson, 1992)
Fibronectin	^{6}RRARVTDATETTITISWRTKTETITGFQVDAIPANG^{41}	b	(Barkalow and Schwarzbauer, 1991)
	^{1906}YEKPGSPPREVVPRPRPGV^{1924}	a	(McCarthy *et al.*, 1990)
	^{1946}KNNQKSEPLIGRKKT^{1960}	a	(Haugen *et al.*, 1990)
Laminin	^{641}RYVVLPRPVCFEKGMNYTVR^{660}	a	(Charonis *et al.*, 1988)
	^{202}RIQNLLKITNLRIKFVK	a	(Kouzi-Koliakos *et al.*, 1989)
	^{3010}KQNCLSSRASFRGCVRNLRLSR^{3032}	a	(Kouzi-Kolaikos *et al.*, 1989)
Vitronectin	^{347}AKKQRFRHRNRKGYR^{361}	a	(de Boer *et al.*, 1992)
			(Kost *et al.*, 1992)
ATIII[e]	^{124}AKLNCRLYRKANKSSKLVSANR^{145}	a,c,d	(Smith *et al.*, 1990)
			(Chang, 1989)
			(Peterson *et al.*, 1987)
			(Smith and Knauer, 1987)
			(Sun and Chang, 1990)
			(Liu and Chang, 1987)
PF4	^{46}KDGRKICLDLQAPLYKKIIKKLLES^{70}	a,c	(Loscalzo *et al.*, 1985)
			(Rucinski *et al.*, 1990)
			(St Charles *et al.*, 1989)
			(Walz *et al.*, 1977)
L-Type C channel	^{219}KGKMHKTCYY^{228}	a	(Knaus *et al.*, 1990)
	^{321}MGKMHKTCYN^{330}	a	(Knaus *et al.*, 1990)
ECGF	^{115}KKHEAKNWFVGLKKGSCKRGP^{144}	c	(Jaye *et al.*, 1986)
Protein C inhibitor	^{264}SEKTLRKWLKMFKKRQLELY^{283}	a	(Pratt and Church, 1992)
90-kDa stress protein	^{362}LYVR^{365}	a	(Itoh and Tashima, 1993)
	^{645}LRQK^{648}	a	(Itoh and Tashima, 1993)
Thrombospondin	^{23}RKGSGRRLVK^{30}	b	(Lawler *et al.*, 1991)
			(Dixit *et al.*, 1986)
	^{77}RQMKKTR^{83}	b	(Lawler *et al.*, 1991)
			(Dixit *et al.*, 1986)
TGF β1	^{23}DFRKDLGWKWIHEPKGYHA^{41}	a	(McCaffrey *et al.*, 1992)
Apo B100	^{3144}LSVKAQYKKNKHRHSI^{3159}	a	(Weisgraber and Rall, 1987)
	^{3352}YKLEGTTRLTRKRGLKLATA^{3371}	a	(Weisgraber and Rall, 1987)

[a]Ability of an analogous heparin binding peptide to bind heparin.
[b]Inability of proteins mutated at the presumed active site to bind heparin.
[c]Inability of chemically modified putative heparin binding residues to bind heparin.
[d]Ability of heparin to protect residues from chemical modification when the heparin binding protein is bound to heparin.
[e]Heparan sulfate also binds this site.

Table 2. Frequencies of amino acids in heparin binding peptides and unrelated proteins

Amino acid	N_f[a]	Number of amino acids[b]	H_f[c]	H_f/N_f[d]
Acidic/amide				
Asx	9.9	38	7.2	0.73
Asp	5.5	14	2.7	0.48
Asn	4.4	24	4.6	1.04
Glx	10.1	38	7.2	0.72
Glu	6.2	25	4.8	0.77
Gln	3.9	13	2.5	0.63
Basic				
Arg	4.7	75	14.3	3.04
His	2.1	12	2.3	1.09
Lys	7.0	82	15.6	2.23
Neutral				
Ala	9.0	22	4.2	0.47
Cys	2.8	15	2.9	1.02
Gly	7.5	31	5.9	0.79
Ile	4.6	21	4.0	0.87
Leu	7.5	46	8.8	1.17
Met	1.7	8	1.5	0.90
Phe	3.5	14	2.7	0.76
Pro	4.6	20	3.8	0.83
Ser	7.1	27	5.1	0.72
Thr	6.0	22	4.2	0.70
Trp	1.1	7	1.3	1.21
Tyr	3.5	22	4.2	1.20
Val	6.9	25	4.8	0.69

[a]N_f, frequency of amino acids in 207 unrelated proteins (Klapper, 1977).
[b]Total number of each amino acid in Table 1.
[c]H_f, frequency of amino acids in heparin-binding sites listed in Table 1.
[d]H_f/N_f, change in amino acid composition in heparin-binding sites compared to random sequences.

tions up to and including that salt concentration. As illustrated in Fig. 2, the relative frequency of the individual amino acids established in the first eluted fraction continued to be similar throughout the elution series. The acidic amino acids, aspartic acid and glutamic acid, could not be analysed individually and were measured and reported as Asx (aspartic acid and asparagine) and Glx (glutamic acid and glutamine). Thus, these two amino acids

Table 3. Frequencies of amino acids in random library and nmoles of amino acids recovered in heparin-Sepharose affinity fractions

Amino acids	L_f[a]	nMol 0.05 M[b]	nMol 0.15 M	nMol 0.3 M	nMol 1 M
Acidic/amide					
Asx	0.119	62.10	46.67	14.66	7.85
Glx	0.117	59.50	46.27	14.75	9.16
Basic					
Arg	0.056	45.27	72.63	31.55	5.84
His	0.056	34.57	29.01	7.48	2.98
Lys	0.060	52.90	71.58	28.43	4.28
Neutral					
Ala	0.052	33.87	27.94	8.41	4.80
Gly	0.056	39.81	38.91	13.24	11.40
Ser	0.050	40.00	33.93	11.28	7.54
Ile	0.062	34.29	27.35	8.21	3.33
Leu	0.052	29.25	23.46	6.97	2.97
Met	0.048	13.58	13.11	6.23	0.20
Phe	0.052	23.85	20.42	6.63	2.44
Pro	0.067	37.52	28.29	8.48	2.82
Thr	0.050	34.50	24.29	7.73	3.55
Tyr	0.050	24.59	21.79	7.13	2.73
Val	0.052	30.67	22.90	6.87	3.06

[a]L_f, frequencies of amino acids in random library.
[b]Peptides were eluted with 0.05 M, 0.15 M, 0.3 M, and 1 M NaCl in 5 mM Na_2HPO_4, pH 7.

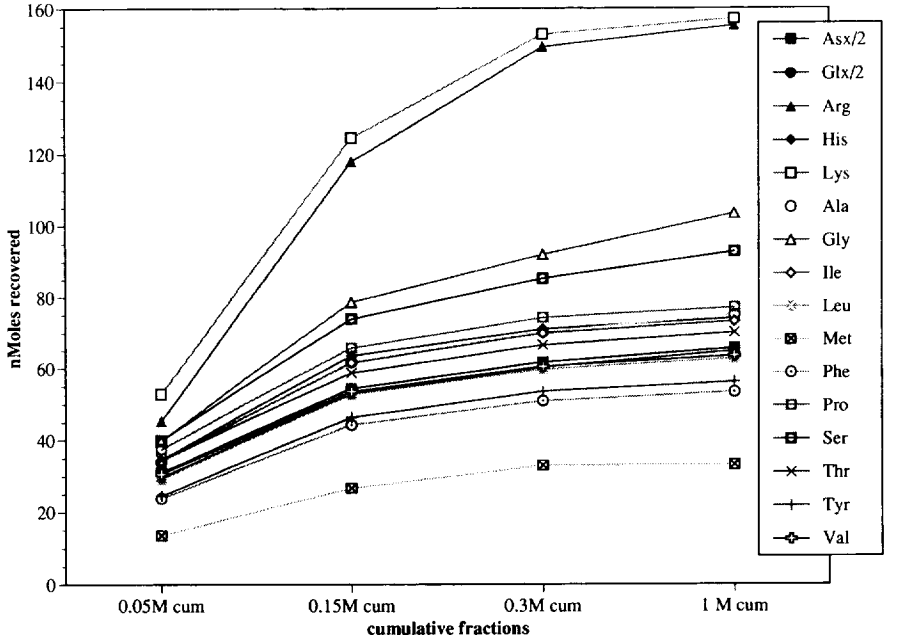

Fig. 2. Cumulative nmol of amino acids in peptides eluted from heparin–Sepharose. Peptides having heparin affinity were eluted at 0.05, 0.15, 0.3 and 1 M NaCl in 5 mM phosphate, pH 7. The cumulative recovery of an amino acid is the sum of recovered nmol of that amino acid in all eluted fractions, up to and including the molarity specified (i.e. 0.3 M cum = nmol[0.05 M] + nmol[0.15 M] + nmol[0.3 M]).

are plotted as Asx/2 and Glx/2 in Fig. 2. Amino acid analysis also did not permit the quantitative comparison of tryptophan and cysteine concentrations in the fractions.

The recovery frequencies (R_f = nmol of specific amino acid/nmol total amino acids) were calculated for each amino acid for each cumulative elution. These recovery frequencies (R_f) were then divided by the corresponding frequency in the original library (L_f) to correct for any variation from equimolarity in the original library. The change in amino acid composition when the peptides were selected based on heparin affinity (Fig. 3) was defined

Table 4. nmol of amino acids recovered in heparin–Sepharose affinity fractions

Amino acids	nMol 0.05 M[a]	nMol 0.15 M	nMol 0.3 M	nMol 1 M
Acidic/amide				
Asx	11.09	2.14	3.43	3.36
Glx	14.36	2.65	6.30	5.45
Basic				
Arg	10.72	1.44	1.48	4.15
His	4.28	0.42	0.85	0.75
Lys	6.90	0.63	1.83	2.68
Neutral				
Ala	6.92	1.22	2.53	2.35
Gly	17.02	2.35	5.79	4.83
Ile	6.16	1.00	1.16	1.23
Leu	6.35	0.94	1.35	1.42
Met	0.00	0.00	0.00	0.00
Phe	5.57	0.73	0.66	0.79
Pro	11.92	0.40	0.83	0.92
Ser	10.52	1.94	6.77	4.06
Thr	4.56	0.83	1.75	1.38
Tyr	5.84	0.73	0.99	1.24
Val	5.76	0.79	1.21	1.15

[a]Peptides were eluted with 0.05 M, 0.15 M, 0.3 M, and 1 M NaCl in 5 mN Na_2HPO_4, pH 7.

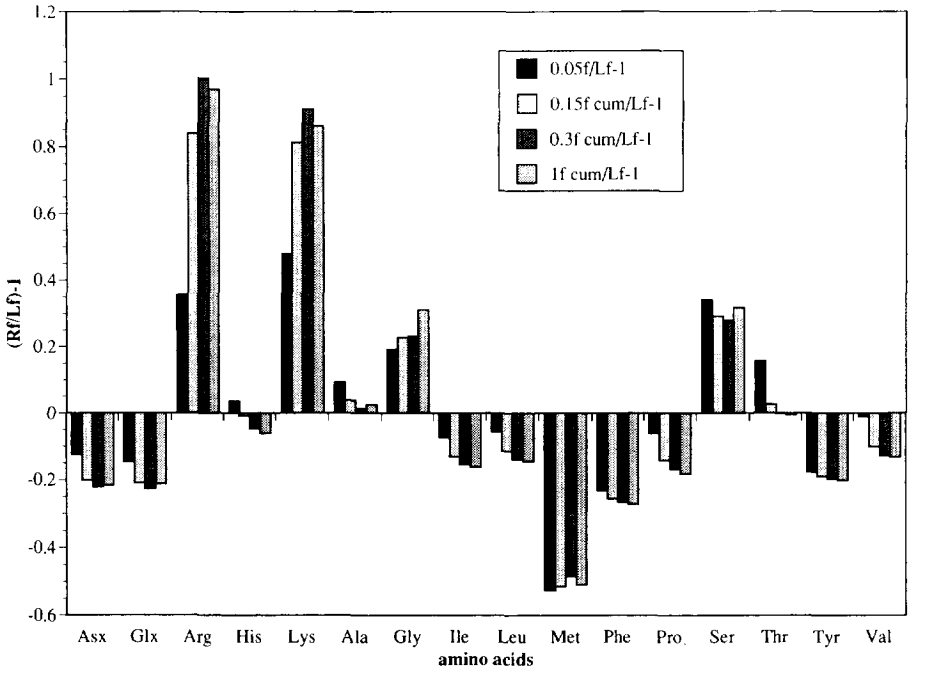

Fig. 3. Changes in corrected cumulative recovery frequencies of amino acids in peptides eluted from heparin–Sepharose. The recovery frequencies (R_f = nmol of specific amino acid/nmol total amino acids in each elution fraction) were calculated for the cumulatively recovered amino acids plotted in Fig. 2. R_f was divided by the corresponding original frequency of the amino acid in the library, L_f (Table 3), to correct for any variation from equimolarity in the original library. $(R_f/L_f) - 1$ is the change in amino acid composition observed when peptides were selected for heparin affinity.

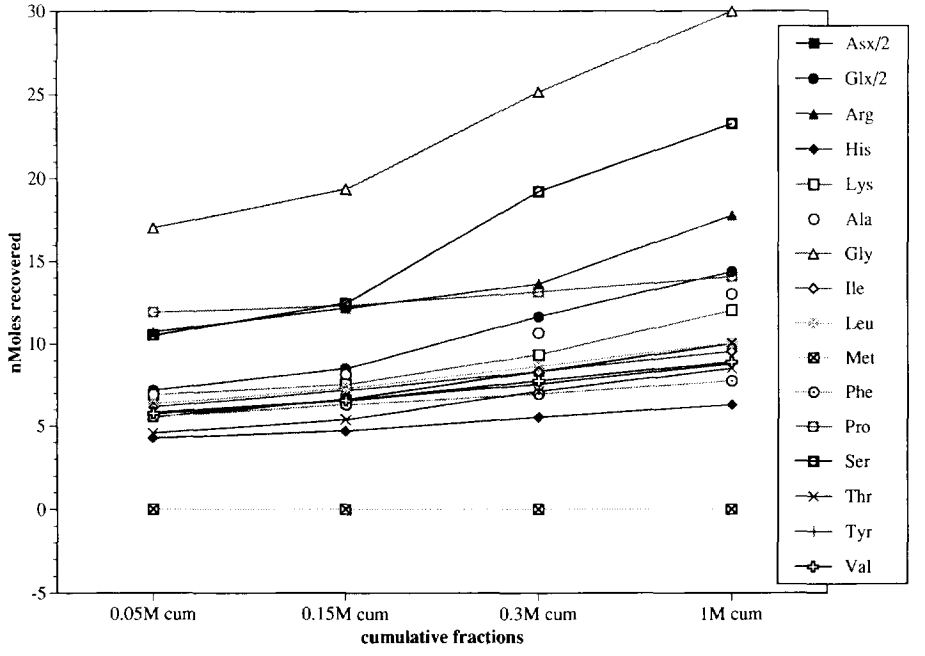

Fig. 4. Cumulative nmol of amino acids in peptides eluted from heparan sulfate Sepharose. Peptides having heparan sulfate affinity were eluted at 0.05, 0.15, 0.3 and 1 M NaCl in 5 mM phosphate, pH 7. The cumulative recovery of an amino acid is the sum of recovered nmol of that amino acid in all eluted fractions, up to and including the molarity specified (i.e. 0.15 M cum = nmol[0.05 M] + nmol[0.15 M]).

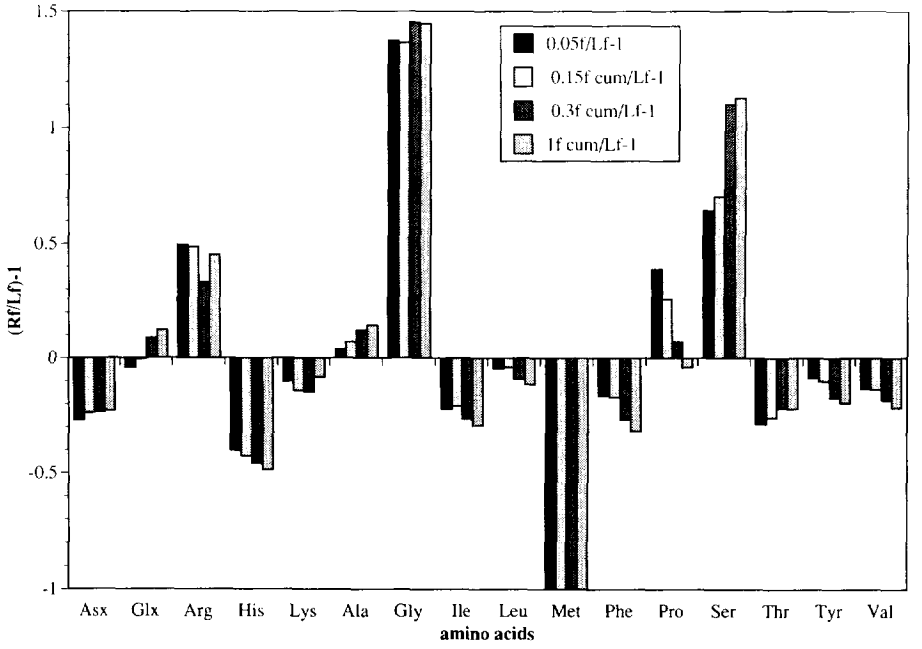

Fig. 5. Changes in corrected recovery frequencies of amino acids in peptides eluted from heparan sulfate Sepharose. The recovery frequencies (R_f = nmol of specific amino acid/nmol total amino acids, Table 4) were calculated for the cumulatively recovered amino acids plotted in Fig. 4. The change in amino acid composition is $(R_f/L_f)-1$, (Table 4) where L_f is the frequency of the amino acid in the library (Table 3).

as (R_f/L_f)-1. When plotted in this way, the enrichment of arginine and lysine is readily apparent. The corrected recovery frequencies (R_f/L_f) for Asx and Glx dropped by 12.5 and 14.8% to 0.875 and 0.852, respectively, in the 0.05 M NaCl fraction where $R_f/L_f = 1.0$ represents no change in the recovery frequency from the original library frequency. These values are consistent with the corrected recovered frequencies observed for Asx and Glx in the known binding sites, reported in Table 2. The only neutral amino acids to increase in corrected frequency were glycine, serine and threonine with (R_f/L_f)-1 = +0.192, +0.342, and +0.175, respectively, in the 0.05 M NaCl fraction. Similar increases were observed in the cumulative recoveries (0.15, 0.3 and 1 M NaCl) of glycine and serine.

Affinity fractionation of random library using heparan sulfate

Heparan sulfate–Sepharose affinity chromatography also selected for less than 3% of the peptides in the library. These affinity selected peptides starting with those eluted at 0.05 M NaCl in 5 mM Na_2HPO_4, pH 7, exhibited a change in amino acid composition when compared with the unfractionated library. This experiment was performed twice giving similar results. A representative experiment is presented in Table 4. Glycine was the dominant amino acid in the eluted peptide fractions. In the 0.05 M NaCl fraction, glycine recovery more than doubled to 17.0 nmol from the predicted equimolar value of 7.4 nmol. Other amino acids that were enhanced by affinity chromatography on heparan sulfate–Sepharose include: serine, arginine and proline. The cumulative recovery of the amino acids plotted in Fig. 4 shows that this initial enhancement in frequency continued throughout the elution series. In the 0.05 M NaCl fraction (Fig. 5), serine, arginine and proline showed a change in corrected recovery frequency of $(R_f/L_f)-1$ = +0.64, +0.50, and +0.39, respectively (Fig. 5). Methionine was not detected in any of the fractions. Histidine was partially depleted in the fractions. The remaining amino acids, including the basic amino acid lysine, were all present at similar corrected recovery frequencies.

DISCUSSION

Previous reports attempting to understand the general features of interactions between proteins and sulfated carbohydrates have described the secondary structural requirements (α-helix and β-sheet) for protein–heparin interactions (Cardin and Weintraub, 1989; Sobel *et al.*, 1992; Margalit *et al.*, 1993). These studies were based primarily on sequence alignment. The studies presented here examined the ability of "non-biased" peptides to interact with heparin and heparan sulfate and provide experimental evidence for the primary structural requirements for a large number of protein/peptide–heparin/heparan sulfate interactions.

Initially, we tested the feasibility of using a random library by examining the binding of [^{35}S]heparin and [^{3}H]heparan sulfate to a resin-immobilized 5-mer random peptide library that was composed of one unique peptide sequence per bead (Lam *et al.*, 1991). This library was synthesized on a scale sufficient to produce approx. 20^5 or 3 million possible sequences, each five amino acids in length. By examining the autoradiograms of [^{35}S]heparin and [^{3}H]heparan sulfate bound to this resin immobilized random peptide library, we obtained a crude estimate of the frequency of sequences with either heparin or heparan sulfate binding affinity in a 5-mer random library. These frequencies suggested that peptides as short as 5 amino acids in length exhibited specific binding to heparin and heparan sulfate. Therefore, we synthesized a 7-mer library containing the most synthetically favorable sequences for subsequent experiments.

Previous heparin binding-site modeling reports have focused on the placement and number of basic amino acids in the binding sequence. Examination of binding sites of known amino acid sequence (Table 2) suggests that the basic amino acids are not equally represented within these binding sites. Surprisingly, they are present in nearly a 3:2:1 ratio (corrected frequencies) of arginine, lysine and histidine, respectively, in the binding sites examined. Therefore, these amino acids may not contribute equally to heparin–protein or heparan sulfate–protein interactions. Indeed, arginine may promote tighter interactions with glycosaminoglycans than lysine or histidine (Fromm *et al.*, 1995).

In the heparin affinity chromatography experiments with the 7-mer peptide library, the majority of peptides did not bind to the column. In the heparin affinity fractions, two of the basic amino acids, arginine and lysine, were recovered in the greatest quantities (Fig. 2). The cumulative amino acid frequencies in the 0.05 and 0.15 M NaCl fractions for arginine and lysine were more than 1.8 times the original library frequencies (Fig. 3). Arginine was present in the eluent at a higher frequency in the heparin binding fractions compared to lysine but the difference was not pronounced. Histidine, the third basic amino acid, was not distinguishable from the majority of the non-basic amino acids. Its corrected cumulative recovery frequency (R_f/L_f) for heparin affinity peptides eluted with 0.05–1 M NaCl was 0.94, which is within 6% of its frequency in the unfractionated library (Fig. 3). This suggests that in the neutral pH environment of this experiment, histidine did not contribute significantly to binding sites with affinity for heparin.

Heparan sulfate affinity fractionated peptides exhibited a greater content disparity between the basic amino acids. At neutral pH, only arginine content was enhanced by affinity chromatography (Fig. 4). Arginine increased to 1.5 times the original library frequency in the first (0.05 M NaCl) eluted fraction (Fig. 5) and remained at 1.3–1.5 times its original frequency for all of the cumulative recoveries from the affinity column. Lysine decreased in frequency after affinity chromatography on heparan sulfate when compared to its frequency in the original library. This decrease in frequency was similar to the decrease observed for six of the neutral amino acids, suggesting that the heparan sulfate column did not select peptides based on lysine content. Clearly, lysine did not appear to play an important role in the interaction of peptides with heparan sulfate. These data, like those for the known heparin binding sites (Table 2), suggest that arginine forges tighter interactions with heparan sulfate than with lysine (Fromm *et al.*, 1995).

The cumulative histidine recovery frequency in peptides that bound to heparan sulfate was approximately half the original frequency in the unfractionated library. Therefore, histidine did not contribute significantly to the interaction of the peptides with heparan sulfate and may have interfered with binding to

heparan sulfate. The depletion of histidine in heparan sulfate binding sites may be due to the lack of protonation (< 10%) of the imidazole ring (p*K*a = 6.04) at neutral pH. Alternatively, the side chain of histidine is shorter than the arginine and lysine side chains and may have experienced more difficulty "reaching" the negatively charged sulfate groups of heparan sulfate or heparin. Although histidine did not appear to be important in binding sulfated polysaccharides at physiologic pH, histidine-containing regions may be expected to bind these molecules at lower pH (Brunden *et al.*, 1993). With protonation of the imidazole ring, which is presumably a requirement to bind anionic carbohydrates, histidine may bind to sulfate groups. We propose that histidine residues could play significant roles in the interaction of heparin and/or heparan sulfate binding proteins with sulfated carbohydrates in the acidic environments such as on the stomach mucosal cell lining surface or in lysosomes.

Heparin is more anionic than heparan sulfate and should bind basic peptides more tightly. This difference in basic peptide binding was observed in the affinity chromatography separations of the 7-mer peptide library. The frequencies for arginine differ between the two types of affinity fractions. Interestingly, arginine was the most prevalent amino acid in the fractions eluted from the heparin affinity column (Fig. 2), but was only the third most prevalent amino acid in fractions eluted from the heparan sulfate affinity column (Fig. 4).

Both glycine and serine increased in frequency in the affinity fractions (Figs 3 and 5). This suggests that these amino acids have chemical properties which contribute to peptide–heparin and peptide–heparan sulfate interactions. Their small side chains should provide minimal steric constraints (maximum flexibility) for peptide alignment with the glycosaminoglycan. Due to decreased sulfation of heparan sulfate compared to heparin, it is likely that fewer basic amino acids are required for maximum interaction. Amino acids that result in the greatest peptide chain flexibility (glycine and serine) when located between basic residues, would likely allow maximum electrostatic interaction with the less frequent anionic sulfate groups of heparan sulfate. The interaction of sulfate groups in sulfated sugars with amides in the backbone of proteins has been demonstrated using X-ray crystallography (Quicho *et al.*, 1987). Therefore, it is also possible that absence of a side chain in glycine permits hydrogen bonding of the sulfated sugar residues to the amides of peptides. The corrected methionine frequency decreased to approx. 0.5 in peptide fractions that bound immobilized heparin. Moreover, methionine was undetectable in fractions that bound heparan sulfate. In contrast, other hydrophobic residues decreased only slightly in frequency. Why methionine is depleted more than other hydrophobic amino acids is unclear.

Proline, due to the ring in its side chain, induces a "kink" in the peptide backbone in those sequences that contain it and commonly occurs in reverse turns. A reverse turn was shown to be important in the interaction of hepatocyte growth factor with heparin (Mizuno *et al.*, 1994). We found that proline residues in known heparin binding sites may occur at the termini of these binding sequences (data not shown) suggesting that a reverse turn may enhance heparin binding by looping the heparin binding site onto the surface of the protein. In the library affinity experiments, the frequency of proline was different in peptides that bound heparin and heparan sulfate (Figs 3 and 5). Peptides that bound heparin showed a decrease in the frequency of proline when compared to the proline frequency in the original library. Conversely, those peptides that bound heparan sulfate exhibited an increase in proline frequency. The role of proline in peptides binding to glycosaminoglycans may be defined by its location relative to the binding site and by the relative importance of two opposing effects of the reverse turn, (A) increased accessibility to adjacent amino acids and (B) decreased flexibility of the backbone.

The amide-containing amino acids, asparagine and glutamine, were not distinguishable from their parent amino acids, aspartic acid and glutamic acid, by amino acid analysis. Although it is assumed that aspartic acid and glutamic acid should decrease peptide binding to sulfated polysaccharides, several authors suggest that asparagine and glutamine may play an important role in the interaction of sulfated polysaccharides with protein and peptides (Zhu *et al.*, 1993; Thompson *et al.*, 1994). In the current study, the frequency of Glx decreased in heparin binding fractions (Fig. 3) and in the known heparin binding sites that we examined (Table 2) but did not

decrease in the heparan sulfate affinity fractions (Figure 5). In the interaction of highly sulfated heparin with protein, electrostatic interactions between basic amino acids and sulfate groups should predominate. Heparan sulfate has a greatly diminished charge density compared to heparin (Lindahl, 1989; Gallagher and Walker, 1985). Consequently, other interactions, such as hydrogen bonding involving an amino acid like glutamine, may play a significant role in the interaction. The increase in the relative importance of the Glx in heparan sulfate binding peptides might have been due to hydrogen bonding of the amide of glutamine to hydroxyls on the monosaccharide ring or possibly hydrogen bonding between the amide of glutamine and the carboxylic acid moiety of glucuronic or iduronic acid. Interestingly, the frequency of the pooled Asx decreased similarly in peptides that bound heparan sulfate or heparin. It is possible that the shorter side chain of asparagine was not long enough to interact effectively via hydrogen bonding in peptides, but may be able to play a significant role in proteins. Alternatively, a decrease in aspartic acid in peptides binding to heparin and heparan sulfate might have masked an increase in asparagine.

The experiments reported here demonstrate that the basic amino acids, arginine, lysine and histidine, are not equally represented in peptides that interact with either heparin or heparan sulfate. This strongly suggests that these basic amino acids do not contribute equally to the interaction of peptides or proteins with heparin and heparan sulfate. Neutral amino acids, such as serine and glycine, which impart flexibility, appear to promote the interaction of peptides with both heparin and heparan sulfate. In contrast, methionine may interfere with the interaction of protein or peptide with heparin or heparan sulfate. Finally, some amino acids such as proline and glutamine might either promote or interfere with the binding of peptides or proteins, depending upon the glycosaminoglycan that is studied. We conclude that heparin and heparan sulfate binding proteins probably do contain specific sequences of basic and non-basic amino acids that foster interactions to control important biological processes.

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