ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 323, No. 2, November 10, pp. 279–287, 1995

Differences in the Interaction of Heparin with Arginine and Lysine and the Importance of these Basic Amino Acids in the Binding of Heparin to Acidic Fibroblast Growth Factor[1]

J. R. Fromm,* R. E. Hileman,* E. E. O. Caldwell,† J. M. Weiler,† and R. J. Linhardt*,[2]
Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242; and †Department of Internal Medicine, Iowa City VA Medical Center and University of Iowa, Iowa City, Iowa 52242

Received July 17, 1995

Although the interaction of proteins with glycosaminoglycans (GAGs) such as heparin are of great importance, the general structural requirements for protein– or peptide–GAG interaction have not been well characterized. Electrostatic interactions between sulfate and carboxylate groups on the GAG and basic residues in the protein or peptide dominate the interaction, but the thermodynamics of these electrostatic interactions have not been studied. Arginine residues occur frequently in the known heparin binding sites of proteins. Arginine is also more common than lysine in randomly synthesized 7-mer peptides that bind to immobilized heparin and heparan sulfate. We have used heparin affinity chromatography, equilibrium dialysis, and isothermal titration calorimetry techniques to further investigate these interactions. A 7-mer of arginine eluted from a heparin-affinity column at 0.82 M NaCl, whereas the analogous 7-mer of lysine eluted at 0.64 M. Similarly, the putative heparin binding site peptide (amino acid residues 110–130) from acidic fibroblast growth factor, which contained four lysine and two arginine residues, eluted at 0.50 M, whereas the analogous peptide with six lysine residues eluted at 0.41 M and one with six arginine residues eluted at 0.54 M. At 25°C in 10 mM sodium phosphate, pH 7.4, carboxy and amino termini blocked arginine (blocked arginine) bound to heparin twice as tightly as blocked lysine as measured by equilibrium dialysis. Similarly, at 30°C in 10 mM sodium phosphate, pH 7.4, and in water, blocked arginine bound 2.5 times more tightly to anions in heparin than blocked lysine. Using titration calorimetry, the enthalpy of blocked arginine and lysine binding to heparin was 1.14 ± 0.24 and 0.45 ± 0.35 kJ/mol, respectively, under identical conditions. Our observations show that blocked arginine- and arginine-containing peptides bound more tightly to GAGs than the analogous lysine species and suggest that the difference was due to the intrinsic properties of the arginine and lysine side chains. The greater affinity of the guanidino cation for sulfate in GAGs is probably due to stronger hydrogen bonding and a more exothermic electrostatic interaction. This can be rationalized by soft acid, soft base concepts.

An understanding of how sulfated polysaccharides interact with proteins or peptides is of great physiologic and pathologic importance. Glycosaminoglycan (GAG)[3]–protein interactions regulate such diverse processes as hemostasis, cell adhesion, lipid metabolism, and growth factor signal transduction (1). However, the nature of the GAG–protein interaction is complex because the GAG often has an unknown primary saccharide sequence (2). The bulk of the literature to date suggests coulombic forces between basic amino acids (positively charged) and anionic (negatively charged) groups on the polysaccharide are of major importance. Cardin and Weintraub (3) examined the amino acid sequences of heparin-binding proteins. This influential paper suggested that certain common primary amino acid motifs fold into α-helices or β-sheets to present a

[1] This work was supported in part by Grants GM38060 and AI22350 from the National Institutes of Health; and a Merit Review Award from the Department of Veterans Affairs. E. E. O. Caldwell is supported by the National Institutes of Health Training Grant T23 HL07344-16. J. R. Fromm is supported by a fellowship from the American Foundation for Pharmaceutical Education.

[2] To whom correspondence should be addressed. Fax: (319) 335-6634.

[3] Abbreviations used: GAG, glycosaminoglycan; aFGF, acidic fibroblast growth factor; *t*-Boc, *t*-butyloxycarbonyl; *p*-MBHA, *p*-methylbenzhydrylamine; FAB, fast atom bombardment.

0003-9861/95 $12.00

linear array of cations which interact with anions on a sulfated polysaccharide. Margalit *et al.* in 1993 (4) followed a similar tact. Starting with known stretches in primary amino acid sequences that bind heparin in GAG-binding proteins, computer modeling suggested a crucial distance of 20 Å between basic amino acids was common in these proteins. To date few experiments have been conducted to define the general structural requirements for GAG–protein interactions.

Growth factor interaction with sulfated polysaccharides appears to be of great physiologic relevance. For example, acidic fibroblast growth factor (aFGF), a cell mitogen, has been implicated in atherosclerorsis and carcinogenesis as well as tumor metastasis (5). It has been proposed that formation of a ternary complex composed of a sulfated polysaccharide, aFGF, and the aFGF receptor may be required for signal transduction (5). We have recently shown that aFGF binds to a specific hexasulfated tetrasaccharide (6). Unfortunately, the amino acids involved in the aFGF–heparin interaction have not been completely defined. Site-directed mutagenesis (7, 8), proteolytic cleavage (9), chemical modification (10), and X-ray crystallization studies (11) implicate a cluster of basic amino acids in the carboxy terminus of the protein (amino acid residues 110–130; Fig. 1). Furthermore, this region of aFGF contains the Cardin and Weintraub heparin-binding-type sequence, XBBBXXBX (3), where B is a basic amino acid (arginine, lysine, or histidine) and X represents any type of amino acid.

Heparin, the most studied of the GAGs, is an alternating polymer of sulfated iduronic or glucuronic acid linked to sulfated glucosamine (Fig. 1). Inspection of the structure of the heparin repeat suggests sulfate or carboxylate groups could interact via electrostatic interactions to cationic residues in a protein or peptide. Hydrogen bonding may also occur with the hydroxyl groups on heparin. Recently, we examined the structural requirements for the binding of a random combinatorial library of 7-mer peptides to heparin- and heparan sulfate-containing matrices (12). In that study, both lysine and arginine (which are fully protonated at physiologic pH) but not histidine appeared to be important for the binding of random 7-mer peptides to heparin, with arginine being slightly more important. Interestingly, arginine appeared to have significantly greater importance, compared to lysine, in the binding of random 7-mer peptides with heparan sulfate. The current study seeks to verify these initial observations and to understand the nature of the preference of arginine for lysine in the binding of sulfated polysaccharides. We demonstrate that a peptide from aFGF binds with high affinity to heparin, suggesting that this peptide contains the heparin-binding site. Analogs of this aFGF binding site peptide, homopolymers of arginine and lysine, as well as blocked arginine and lysine were also studied to demonstrate further the differential importance of basic amino acids in the recognition and binding of sulfated polysaccharides. Taken together, these studies suggest that these two basic amino acids take on different roles in the interaction of GAG with protein or peptide. Such information should facilitate understanding of biologically relevant GAG–protein interactions as well as the design of therapeutics to alter this interaction.

EXPERIMENTAL PROCEDURES

Materials

Blocked lysine, Ac-lysine-$NH_2 \cdot$ HCl (*N*-(α-acetyl)-L-lysine amide, hydrochloride salt), and blocked arginine, Ac-arginine-$NH_2 \cdot 1.1$ AcOH $\cdot 0.7$ H_2O (*N*-(α-acetyl)-L-arginine amide, acetate salt), were purchased from Bachem Bioscience Inc. (Philadelphia, PA). Blocked lysine was used without further purification. Blocked arginine was converted to the HCl salt by chromatography on anionic Dowex 1×2 (Sigma, St. Louis, MO). The structure and purity of both blocked amino acids were assessed using high-resolution 500 MHz ^{1}H NMR. The spectra obtained for both were consistent with the aforementioned structures and showed that blocked lysine and arginine were greater than 99 and 94% pure, respectively. Heparin, sodium salt (165 U/mg), was obtained from Celsus Laboratories (Cincinnati, OH). Prior to use, the heparin was exhaustively dialyzed against 3×1000 vol of distilled, deionized water using either 7000 molecular weight cutoff (for equilibrium dialysis experiments) or 3500 molecular weight cutoff (for calorimetry experiments) membranes from Spectrum Medical Industries (Los Angeles, CA) and freeze-dried. Prepacked heparin–agarose columns were purchased from Sigma Chemical Co. Equilibrium dialysis cells were from Science Ware (Fisher Scientific, Itasca, IL). *o*-Phthaldehyde (OPA) solution was from Pierce (Rockford, IL). RNase A (type XII-A from bovine pancreas) and 2′-CMP were purchased from Sigma Chemical Co. *t*-Butyloxycarbonyl (*t*-Boc) amino acids were from Advanced ChemTech (Louisville, KY). Resin, *p*-methylbenzhydrylamine (*p*-MBHA) was from Colorado Biotechnology Associates (Denver, CO). Trifluoroacetic acid was from Halocarbon Products (Augusta, SC). Reversed-phase C18 μBondapak columns were from Waters Chromatography (Milford, MA). All other reagents were from either Fisher Scientific (Pittsburgh, PA) or Aldrich Chemical (Milwaukee, WI).

Methods

Design of the aFGF heparin-binding domain peptide. Analysis of the aFGF structure was performed using SYBYL (ver. 6.1) molecular analysis software from Tripos, Inc. (St. Louis, MO) on a Silicon Graphics Elan workstation.

Peptide synthesis and purification. Peptides were synthesized on *p*-MBHA resin using standard *t*-Boc solid-phase chemistry (13, 14) or the tea-bag technique, in which the *p*-MBHA resin was compartmentalized in polypropylene bags (15). During each coupling cycle, the bags were pooled for the deblocking and base washing steps and were only separated for the coupling reactions. All amino acids were N-terminal blocked with *t*-Boc. The side chains were protected as arginine (*N*-guanidinotoluenesulfonyl), Cys (*S*-4-methylbenzyl), His (N^{im}-benzyloxymethyl), lysine (*N*-ϵ-2-chlorobenzyloxycarbonyl), Ser (*O*-benzyl), Thr (*O*-benzyl), and Tyr (*O*-2,6-dichlorobenzyl). After the final deblocking step, the peptides were cleaved from the resin and their side chains were deprotected using a standard HF/anisole procedure (16). As many as 10 intact bags of resin were cleaved simultaneously in a compartmentalized reaction vessel from Multiple Peptide Systems (San Diego, CA). Ethyl acetate was used to remove the residual anisole before the peptides were extracted with 15% acetic

acid. The resulting peptide preparations contained C-terminal amides. After lyophilization, the crude peptides were analyzed by reverse-phase HPLC (Waters μBondapak C18, 3.9 × 300 mm) starting at 1 ml/min 0.1% trifluoroacetic acid in water and using linear gradients of 0 to 100%, 0.04% trifluoroacetic acid in acetonitrile. Preparative purification used similar gradients and a Waters μBondapak C18 column (19 × 150 mm).

Confirmation of peptide identity and purity. Fast atom bombardment (FAB) mass spectrometry was performed by the High Resolution Mass Spectrometry Facility of the Department of Chemistry at the University of Iowa. A ZAB HF VG analytical mass spectrometer was used to identify the molecular weight and to confirm the complete deprotection and purity of the peptides in either a glycerol or a thioglycerol matrix. Analysis of these mass spectral data also gave a partial sequence for each peptide that was consistent with its structure.

Heparin–agarose affinity elution of synthetic peptides. Prepacked heparin–agarose columns (2.5 ml, 750–1000 μg heparin/ml gel) were first washed with 5 column volumes of phosphate buffer (5 mM sodium phosphate buffer, pH 7.4) containing 2 M NaCl and then equilibrated with 10 column volumes of phosphate buffer. Peptide (70 μg, measured spectroscopically) was loaded onto the column, the column was washed with 10 column volumes of phosphate buffer and fractions containing nonbinding material were collected. The absorbance of these fractions at 274 nm for the aFGF analog peptides or 279 nm for the R_7W and K_7W peptides (see Table I for structures) was measured to verify peptide binding. The column was then eluted with a linear 0 to 2 M NaCl gradient (35 ml) in phosphate buffer. Fractions (0.7 ml) were collected and the elution profile of the peptide was determined by monitoring the absorbance. Salt concentration required for peptide elution was quantified by measuring conductivity (Solution Analyzer, Amber Scientific, San Diego, CA) of elution fractions diluted 40 times with water and comparing to a standard curve made from conductivity measurements of solutions of known salt concentration.

Coelution of R_7W and K_7W peptides from heparin agarose. Peptides (35 μg of R_7W and 35 μg of K_7W) were mixed in phosphate buffer and the solution was eluted in a manner identical to the single peptide elution experiments. Fractions having an absorbance value greater than 0.01 at 279 nm were assayed separately by the lysine specific OPA assay (17) and the arginine-specific Benzoin assay (18). Note that the equimolar peptide solution did not saturate the column as evidenced by the absence of peptide in the phosphate buffer wash prior to elution.

Equilibrium dialysis measurements of heparin with blocked lysine and arginine. This technique permits the determination of dissociation constants for large molecules, such as heparin, binding to small ligands, such as blocked amino acid. Heparin was placed on one side of a membrane (MWCO 3500), with pores too small to allow the heparin to pass. Amino acid ligand, which could flow freely through the membrane, was placed on both sides of the membrane at identical concentration. After the system reached equilibrium the concentration of ligand on both sides of the membrane was measured. Preliminary experiments verified that: (a) 3 days was sufficient for equilibrium to be obtained, (b) in the absence of heparin, blocked amino acids concentrations on the two sides were identical, and (c) no heparin moved across the membrane as determined by carbazole assay (19).

Solutions (7.5 ml each) of blocked amino acid in 10 mM sodium phosphate, pH 7.4, buffer and amino acid and heparin in 10 mM sodium phosphate, pH 7.4, buffer were prepared separately. The two solutions were then loaded in the 10 ml per chamber equilibrium dialysis cell and shaken gently (50 rpm) at 25 or 30°C for 3 days. Solutions were then removed from the apparatus and either assayed using the OPA (lysine-containing experiment) or the Benzoin assay (arginine-containing experiment). For the lysine-containing experiments it was also necessary to determine heparin concentration by the carbazole assay (19) as heparin contains a small amount of OPA-reactive free amino groups. Heparin concentrations were also confirmed using carbazole assay in the arginine experiments. In the arginine experiments, blocked amino acid concentrations ranged from 3.36 to 33.6 μg/ml and heparin from 500 μg/ml to 1.5 mg/ml. In the lysine experiments, blocked amino acid concentrations ranged from 3.36 to 50.4 μg/ml and heparin from 500 μg/ml to 1.5 mg/ml. The maximum ratio of arginine residues to heparin chains was 4.2, while the maximum ratio of lysine molecules to heparin chains was 6.3. Consequently, all equilibrium dialysis experiments were conducted under nonsaturating conditions.

A long polymer such as heparin with n identical independent binding sites for ligand A can be described mathematically. Using the definition of the per site dissociation constant K_d and mass balance equations, the following simple relationship may be derived.

$$K_d = (n - R)A/R,$$

where $R = (A_T - A)/H_T$, A is the concentration of free blocked amino acid, A_T is the total concentration of blocked amino acid, and H_T the total concentration of heparin used in the experiment. An n of 100 was selected based both on the known charge of heparin and the results obtained using titration calorimetry.

Titration calorimetry. All calorimetric data were collected using a Model 4209 Hart Scientific Microtitration Calorimeter (Pleasant Grove, UT). The voltage to the instrument was regulated with a Citadel power conditioner, Model LC630, from Best Power Technology, Inc. (Necedah, WI) to minimize noise due to voltage fluctuations. A digitally controlled external water bath (Model 9109, Polyscience, Niles, IL) was set 5°C less than the operating temperature for data collection. Electronic calibration was carried out using 10–1000 μJ pulses at 200-s intervals to the reaction cell containing 1 ml of water. Chemical calibration was carried out by comparing the observed enthalpy of ionization for tris-(hydroxymethyl) aminomethane to the theoretical value (20) using 10 5-μl injections of 1 mM HCl into 1 ml 250 mM (Tris) base at 200-s intervals. This resulted in a small (1%) correction to the electronic calibration parameter. In a separate experiment, the calibration parameter was chemically verified by titrating RNase A with 2′-CMP; the observed K_a, ΔH, and n values were within 1–10% of the previously published values (21). For all titrations, 20 5-μl injections of the smaller ligand (amino acid) were pipetted automatically into the reaction cell containing 1 ml of the larger molecule (heparin) at 200-s intervals from a 100-μl syringe while stirring at 75 rpm. In all experiments, 10 mM sodium phosphate buffer (pH 7.4) was used at 25°C and the thermal reference cell contained 1 ml water. Integration of the thermogram peaks was carried out using the software supplied with the calorimeter (Hart Scientific). The total corrected heats were obtained after subtraction of the control heats of dilution for the ligand at each injection. In all control experiments, the ligand was simply diluted into buffer with the large molecule omitted. The corrected heats were fitted using a nonlinear least-squares algorithm which minimized the sum of squared residuals while varying ΔH, n, and K_a as previously described (21–23).

RESULTS

Design of the aFGF heparin binding domain peptide and analogs. To locate the heparin-binding site on aFGF for heparin, the crystal structures of the protein and analogs were examined using molecular graphics (24). A cluster of basic residues on the protein surface from amino acid residue 110–130 was observed (Fig. 1). This is also the region implicated in heparin binding by site-directed mutagenesis (7, 8), chemical modifica-

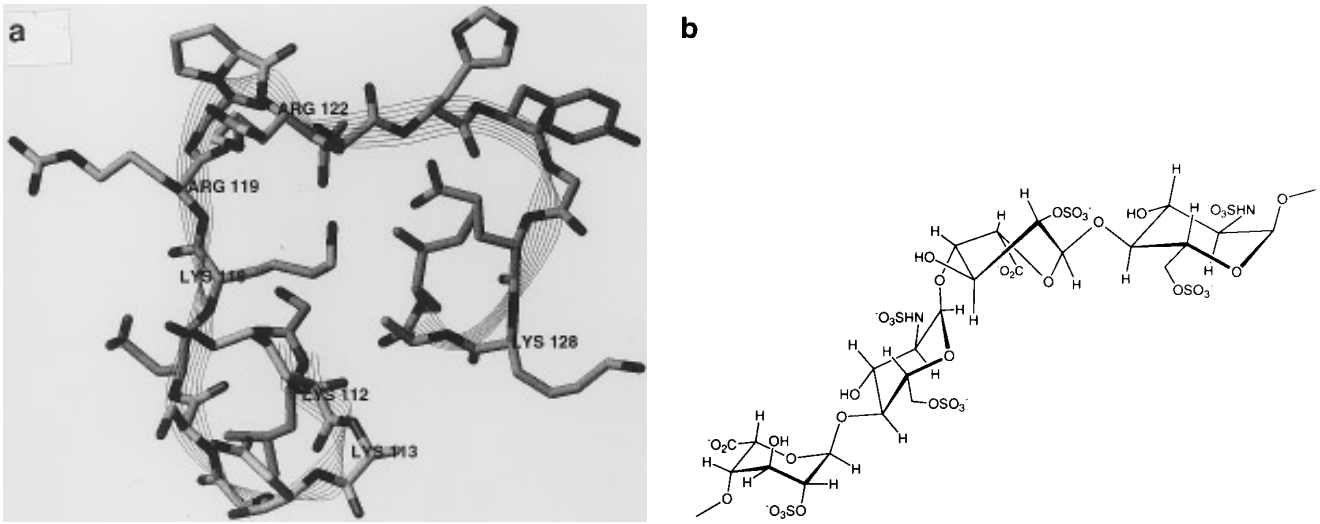

FIG. 1. Putative heparin-binding site in aFGF and the aFGF binding site in heparin (drawn to scale). (a) aFGF(110–130) is taken from the crystal structure (24). The basic residues are labeled. Lys 113 was not localized and Cys 117 is shown with its lone pairs of electrons. (b) A tetrasaccharide sequence in heparin known to bind aFGF (6) is shown drawn in the conformation suggested in solution NMR studies (2). One uronic acid is in the skew-boat form, the other in the chain form; both are found in equal amounts in solution.

tion experiments (10), and cocrystallization of sucrose octasulfate (SOS) with aFGF (11). A heparin tetrasaccharide, corresponding to the heparin sequence (Fig. 1), determined to be the minimum aFGF-binding site in heparin by footprinting experiments (6), also protects aFGF from inactivation at this site (25). A peptide corresponding to the aFGF sequence from amino acid residue 110 to 130 (denoted aFGF(110–130)) was synthesized and its affinity for heparin–agarose was assessed. aFGF(110–130) eluted at 0.50 M (Table I) strongly suggesting that this peptide contained the heparin-binding region of native aFGF.

To determine the relative importance of arginine and lysine in the binding of peptide and protein to sulfated oligosaccharides, analog peptides of the aFGF-binding site peptide aFGF(110–130) were synthesized and their affinities for heparin–agarose were assessed (Table I). The aFGF analog peptide with six lysines (aFGF-K) eluted at 0.41 M NaCl, whereas the aFGF-binding site peptide with six arginines (aFGF-R) eluted at significantly higher NaCl concentration, 0.54 M. The data suggest that among these three peptides, arginine residues promote tighter binding of these peptides to heparin than lysine residues.

Affinity fractionation and coelution of R_7W and K_7W peptides. Defined homopolymers of arginine and lysine were synthesized and affinity for heparin was measured by heparin–agarose affinity chromatography to verify that the higher affinity of the arginine-containing aFGF analog peptides was not the result of a peculiarity of aFGF analog peptides. K_7W eluted at 0.64 M NaCl and R_7W eluted at 0.82 M NaCl (Table I) confirming that arginine-containing peptides eluted at higher salt concentration than the analogous lysine-containing peptide. R_7W and K_7W peptides were similarly analyzed in an equimolar mixture under conditions that would not saturate the column. The peptides were eluted with a 0 to 2 M NaCl gradient in 5 mM sodium phosphate buffer. Fractions containing peptide (by absorbance at 280 nm) were assayed by a lysine-

TABLE I

Affinity of Peptides for Heparin Agarose

Peptide	Sequence	Salt concentration (M) required for release
aFGF(110–130)	GLKKNGSCKRGPRTHYGQKAI	0.50
aFGF-R	GLRRNGSCRRGPRTHYGQRAI	0.54
aFGF-K	GLKKNGSCKKGPKTHYGQKAI	0.41
R_7W	RRRRRRRW	0.82
K_7W	KKKKKKKW	0.64

TABLE II

Equilibrium Dialysis for the Determination of the Dissociation Constants for the Binding of Blocked Amino Acids to Heparin

Sample	Condition[a]	$K_d \pm$ SD (mM)[b]	$K_{d,Lys}/K_{d,Arg}$
Blocked lysine	Water, 30°C	0.75 ± 0.018	2.42
Blocked arginine	Water, 30°C	0.31 ± 0.06	
Blocked lysine	Buffer, 30°C	39 ± 14	2.05
Blocked arginine	Buffer, 30°C	19 ± 5	
Blocked lysine	Buffer, 25°C	80 ± 53	2.76
Blocked arginine	Buffer, 25°C	29 ± 12	

[a] Buffer in all cases is 10 mM sodium phosphate, pH 7.4

[b] K_d measurements represent three to six replicate trials for each blocked amino acid under each condition. SD is standard deviation.

specific assay (OPA) and arginine-specific assay (Benzoin assay; see Fig. 2). Clearly the lysine-containing peptide eluted before the arginine-containing peptide.

Equilibrium dialysis measurements of K_d for blocked amino acids. A still unresolved question is whether the tighter binding of arginine than lysine-containing peptides is due to an intrinsic property of the amino acid side chains or simply due to a property of arginine-containing peptides (such as peptide conformational differences). To address this question, dissociation constants of amino acid for heparin were measured. The amino acids used in the study were amidated at the carboxy terminus and acetylated at the amino terminus so as to resemble an amino acid in a peptide chain and represent affinity of the amino acid side chains for heparin without the influence of the peptide backbone or the amino and carboxy functionalities. Experiments were first conducted in water at 30°C to eliminate any effect of ions in solution on K_d. Binding constants were also measured at 25 and 30°C in 10 mM phosphate buffer, pH 7.4. Data from the experiments are shown in Table II. Under all conditions, blocked arginine bound to heparin from 2.1 to 2.7 times greater than the blocked lysine suggesting the higher affinity of arginine for heparin was due to a tighter interaction of the arginine side chain with anionic groups in heparin. Blocked arginine and lysine bound more tightly in water than in phosphate buffer. Although temperature increases affinity for both lysine and arginine for heparin, this result may not be significant because of the overlap of standard deviation.

Isothermal titration calorimetry. The thermodynamics of the blocked arginine and lysine interaction with heparin was also characterized using isothermal titration calorimetry. Titration calorimetry measures heat released upon ligand binding. One experiment affords simultaneous characterization of ΔH, K_a, and n. A representative titration of heparin with arginine and

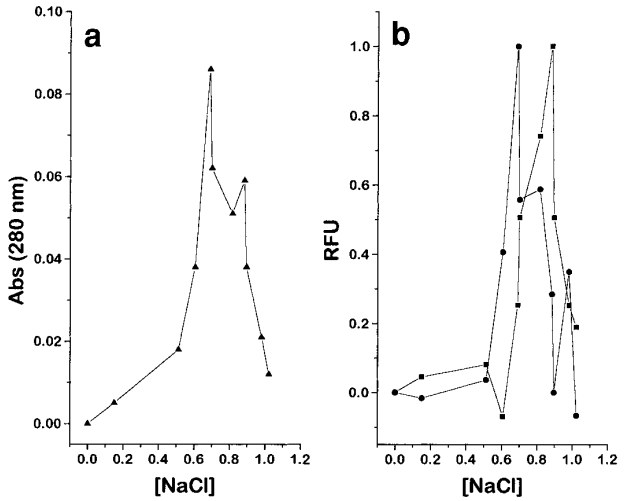

FIG. 2. Elution of an equimolar mixture of K_7W and R_7W from a heparin–agarose affinity column using a linear salt gradient. (a) The elution profile (▲) measured by absorbance at 280 nm as a function of salt concentration. (b) The elution profile of R_7W (■) and K_7W (●) measured by fluorescence as a function of salt concentration using arginine and lysine specific assays. The maximum relative fluorescence of both curves was arbitrarily set to 1.0.

the corresponding blank heats of dilution is shown in Fig. 3a. Integration of the peaks yields the heats released per injection (Fig. 3b). The data were fitted to a

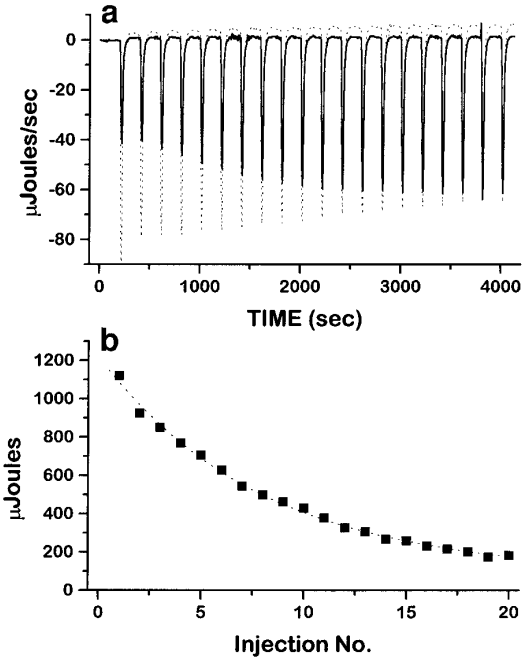

FIG. 3. Isothermal titration calorimetry as a measure of the binding of blocked arginine to heparin. (a) A representative titration of heparin with blocked arginine. The solid line is blocked amino acid titrated into heparin in buffer. The dotted line is blocked amino acid titrated into buffer (control). (b) The binding isotherm, heat released as a function of injection number.

TABLE III

Summary of the Observed Interaction Parameters for Blocked Arginine and Lysine with Heparin[a]

	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol)	K_a (M^{-1})	n
Arginine	−1.14 ± 0.24	−11.0 (−10.6 to −11.3)	33.1	84.7 ± 11.3	97.6 ± 15.1
Lysine	−0.45 ± 0.35	Ind[b]	Ind	Ind	Ind

[a] Five and eight separate trials were completed for the arginine and lysine data, respectively.
[b] Indeterminant due to insufficient heat of interaction.

nonlinear function which floats ΔH, K_a, and n (21–23). The observed independent variables ΔH, K_a, and n, as well as the calculated dependent variables ΔG and ΔS are shown in Table III for the interaction of blocked arginine with heparin. The arginine values represent five separate trials using heparin concentrations ranging from 96 to 124 μM titrated with arginine concentrations ranging from 288 to 372 mM. The reported ΔH for blocked lysine binding to heparin represents eight separate trials using heparin and lysine concentrations ranging from 54 to 150 μM and 146 to 487 mM, respectively. Wiseman and co-workers (21) reported that a value greater than 1 for the product of K_a and the large molecule concentration, termed c, was necessary to obtain binding isotherms having accurate K_a and n values. Characterization of blocked lysine binding to heparin was difficult to obtain by this method ($c < 0.02$) as indicated by the large standard error in the observed ΔH. Generally, however, accurate measurement of the enthalpy of binding ΔH can still be made for weak interactions, i.e., $c < 1$ (21). The interaction of blocked arginine to heparin released 2.5 times more heat than did the interaction of heparin with blocked lysine. Experiments in which the addition of heparin and blocked amino acid were reversed (amino acid in the cell and heparin in the dropping syringe) did not yield the same observed values for ΔH, K_a, and n. This may have been due to the ability of heparin, a polyanion, to impart order on the structure of water resulting in the very large heats of dilution observed for heparin.

DISCUSSION

Electrostatic interactions are of paramount importance in biological and chemical systems. These forces in part define the stability of large molecules such as proteins and play an important role in the recognition of biological molecules. Indeed, coulombic forces appear to dominate the interaction of sulfated polysaccharides with proteins (1). This study has elucidated the nature and magnitude of these interactions. Because there are three times as many sulfates as carboxylates in the heparin polymer, the values describing these interactions (Tables I, II, and III) are dominated by the interaction of amino acid with sulfate anion. Because of the heterogenous nature of heparin, measured affinities of ligands for heparin are in reality average values. The observed affinity of arginine-containing peptides for heparin was greater than that of lysine-containing peptides as measured by affinity chromatography. Similarly, the affinity of blocked arginine for heparin was greater than blocked lysine's affinity as measured by equilibrium dialysis and microtitration calorimetry. Arginine bound heparin approximately 2.5 times more tightly than lysine under a variety of conditions (water and 10 mM sodium phosphate; 25 and 30°C) and the arginine–heparin interaction released 2.5 times more heat than lysine–heparin, despite the fact that at the pH used, both arginine and lysine have an identical charge of +1. A linear relationship exists between log K_d vs log [Na^+] for positively charged ligands binding to heparin (26). Using our data for K_d measured for blocked arginine and lysine binding to heparin, we estimate $K_{d,Lys} = 0.79$ M, $K_{d,Arg} = 0.44$ M at 0.1 M [Na^+] and 30°C. Therefore $K_{d,,Lys}/K_{d,Arg} = 1.8$ suggesting that for the blocked amino acids, arginine binds more tightly than lysine under physiologic ionic strength conditions.

Similar results were obtained when peptide–heparin interactions were examined. The peptide R_7W bound more tightly to heparin than did K_7W, despite the fact that both having an identical charge of +7. This difference is not limited to polyarginine or polylysine systems; aFGF analog binding-site peptide studies showed that aFGF-R bound more avidly than the native aFGF binding-site peptide (aFGF(110–130)), despite the identical number and distribution of cationic residues. In contrast, an aFGF-K bound less tightly than aFGF(110–130). Together, the results demonstrate that the tighter interaction of blocked arginine amino acid and arginine-containing peptides with GAGs is due to a structural feature in the basic side chain that enhances binding; consequently, the greater affinity of arginine-containing peptides cannot be due to conformational differences of these peptides compared to lysine containing peptides.

Demonstration that aFGF(110–130) binds with relatively high affinity adds further evidence that this region is the heparin-binding site. Chemical modification and site-directed mutagenesis experiments have impli-

cated Lys118 in the binding of heparin (10). The crystal structure of a SOS–aFGF complexes suggests that the heparin-binding site encompasses a linear protein sequence from residues 112 to 127 (11). Because SOS was observed to bind only a portion of the heparin-binding site, it is reasonable that residues 128 to 130 contribute to the site. Note that residue 128 is a lysine residue. Our laboratory has previously suggested this region contains the heparin-binding site based on its protection against inactivation by a heparin tetrasaccharide (25). A tetrasaccharide sequence in heparin is also sufficient in size to occupy the entire binding site (Fig. 1) and aFGF protects this tetrasaccharide sequence in footprinting experiments (6). With this improved understanding of the interaction of heparin and aFGF, the potential for the formation of a ternary complex of sulfated oligosaccharide, aFGF, and receptor may now be examined in greater detail.

Based on the structural homology of the phosphoryl and sulforyl groups, our current understanding of the interaction of phosphoryl anions with proteins may provide insight into the sulforyl anion/amino acid cation interaction. In phosphoryl/cation interactions, arginine appears to play a more important role than lysine or histidine. Conserved protein domains that bind phosphotyrosine (SH2 domains) contain more arginine than lysine residues (27, 28), presumably due to the avid interaction of arginine with phosphoryl anions, compared to the interaction of lysine with the phosphoryl anion. Of the three invariant amino acids in SH2 domains (thought to interact directly with the phosphoryl group), two are arginine and one is a histidine residue. Riordan and co-workers (29) examined the substrate binding sites (phosphate binding) of the glycolytic enzymes with 2,3-butadione, which chemically modifies arginine residues. Based on these studies, they concluded arginine was more important than lysine in the binding of phosphate dianion and speculated that arginyl residues play key roles in anion recognition.

The interaction of heparin with homopolymers of arginine and lysine have been examined previously. An important set of papers by Gelman and Blackwell and colleagues (30–32) demonstrated polyarginine α-helix denatured at a higher temperature when binding to GAGs than an analogous polylysine polymer (30). The authors, however, attributed this difference to the larger arginine side chain more effectively "reaching" the anionic groups of the GAG rather than a tighter interaction of the arginine side chain with GAG.

Our laboratory has also studied the importance of primary protein structure in the interaction of protein with GAGs (12). By examining the frequency of amino acids of proteins in sites known to bind heparin as well as combinatorial peptides with high affinity for heparin and heparan sulfate agarose, we observed a preference of arginine over lysine. Histidine was also shown to be unimportant in the binding of GAGs by protein and peptide at pH7. The current study helps to explain the nature of this preference.

The observed K_d values for both the blocked arginine and blocked lysine interaction with heparin indicate very weak binding (29 and 80 mM, respectively at 25°C in 10 mM phosphate buffer, pH 7.4). However, these values are as expected for a single cation–anion electrostatic interaction. The interactions between two large molecules can be described by a multiplicity of single interactions, possibly of varied types (i.e., hydrogen bonding, electrostatic interactions), occurring at points where the molecules contact. Due to the initial binding of their individual interactions, the effective concentration becomes increased for the remainder of the individual interactions describing the overall interaction (33). Thus, although a single electrostatic interaction may be weak, a series of electrostatic interactions between a protein and heparin forges a tight interaction. In addition, other interactions undoubtedly occur (i.e., hydrogen bonding, hydrophobic interactions) that would also promote tighter interaction.

Why does the guanidino group of arginine interact more tightly than the ammonium cation of lysine? First, differences in hydrogen bonding may be an important factor. Molecular modeling studies clearly define the differences between ammonium and guanidinium cations in hydrogen bonding to sulfate groups (Fig. 4). Guanidino groups can form five hydrogen bonds in which the N—H · · · O bond angle approaches the ideal of 180° or two hydrogen bonds in which this crucial angle is almost exactly 180°. A similar hydrogen bonding interaction, observed in the crystal structure of methyl guanidinium–dihydrose phosphate was reported by Cotton and co-workers (34). Ammonium cations can form seven hydrogen bonds, but the N—H · · · O bond angle approaches approximately 120° suggesting that the hydrogen bonds formed would be significantly weaker. Alternatively, the ammonium cation could form one hydrogen bond in which this angle is 180°.

Second, the guanidino cation rather than the ammonium cation may form an inherently stronger electrostatic interaction with the sulfate anion. This is rationalized based on Pearson's concept of soft acid, soft base interactions (35). A large soft base should interact more tightly with a large soft acid than with a small hard acid (and vice versa). Consequently, the relatively large sulfate anion (soft base) should bind more avidly with the large guanidino cation (soft acid) than the ammonium cation (hard acid). Note that the more negative ΔH of interaction of arginine with heparin (more heat released on binding) is accord with either a hydrogen bonding or soft acid–soft base arguments.

Clearly, arginine residues bind anions (predominately sulfates) on GAGs more tightly than lysine resi-

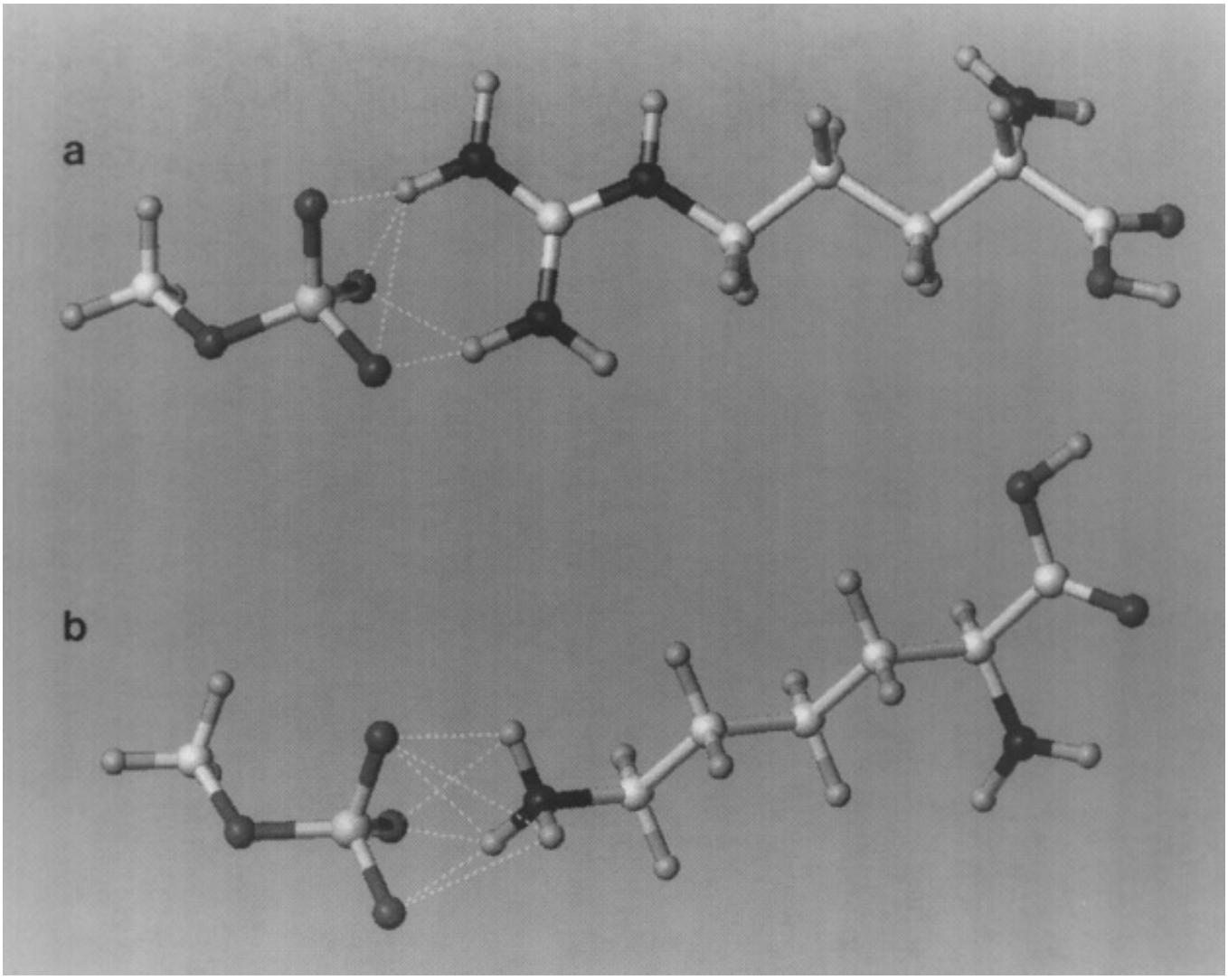

FIG. 4. Ion-pairing of methyl sulfate with arginine and lysine. (a) Methyl sulfate anion (left) interacting with the guanidino cation of arginine (right). (b) Methyl sulfate anion (left) interacting with the ϵ-ammonium group of lysine (right). The dotted lines are computer-calculated hydrogen bonds.

dues. We have previously shown, however, that although the normalized frequency of arginine residues in known heparin-binding sites is greater than lysine, all of the basic residues in these sites are not arginine residues (12). What is the physiologic advantage of including lysine residues that promote a less avid interaction? Lysine residue side chains are flexible (i.e., in the crystal structure of aFGF lysine 113 cannot be localized (Fig. 1)) perhaps allowing the cation to more readily ion pair with its anion. In addition, it may be that through the combination of arginine and lysine (and presumably other non-basic residues) the affinity of a given protein for GAG has been tailored to its physiologic role. For example, aFGF binds to the glycosaminoglycan chain of a proteoglycan and its tyrosine kinase receptor to promote signal transduction (5). Perhaps if aFGF bound too tightly to the GAG side chains of the proteoglycan, the aFGF's signal would be spuriously transduced. Conversely, if aFGF bound the GAG side chain too weakly, not enough signal would be propagated. Evolution may have "fined tuned" the affinity of aFGF for GAG by selecting for a heparin binding site of four lysine and two arginine residues.

The tighter interaction of arginine than lysine with heparin may have ramifications in the development of therapeutic agents. For example, aFGF-R would be expected to be a better antagonist of aFGF binding to heparin *in vitro* than aFGF(110–130). Likewise, peptidomimetic drugs designed to interact avidly with phosphate by mimicking an SH2 domain should have highest activity if arginine residues are employed.

The results presented by Riordan and co-workers (29) suggest strongly that arginyl residues play a unique role in nature in anion recognition. Our results support this hypothesis. The large diffuse (soft) is ideally suited to interact with large (soft) bioanions, such as sulfate and phosphate. Indeed it has been suggested that arginine appeared later evolutionarily to perform important biological functions (29, 36, 37). We propose that one of those functions is to interact with sulfate residues. Studying arginine may provide key insight into the nature of the interactions of large molecules.

Note added in proof. After submission of this manuscript, Mascotti and Lohman reported the thermodynamics of heparin–peptide interaction. They found that an arginine containing peptide bound tighter to heparin than a lysine containing peptide. (Mascotti, D. P. and Lohman, T. M. (1995) *Biochemistry* **34,** 2908–2915).

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