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Relative retention of the fibroblast growth factors FGF-1 and FGF-2 on strong cation-exchange sorbents

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Abstract

The isocratic retention of two heparin-binding fibroblast growth factors, FGF-1 (acidic FGF) and FGF-2 (basic FGF), was compared on a set of six preparative strong cation-exchange adsorbents. The FGFs comprise a solute pair that are structurally equivalent, yet differ in protein parameters of potential importance in cation-exchange chromatography, such as isoelectric point, net charge, and the number and distribution of basic amino acids. The cation-exchange adsorbents comprise a diverse set of materials in common use for protein purification, with physical and chemical properties that have been characterized and described previously. Isocratic k' values for the two proteins obtained on each adsorbent at several different [NaCl] are compared with one another and with corresponding data for hen egg lysozyme, which is also strongly retained on cation-exchangers. Of the six adsorbents examined, three showed strong retention of both FGFs, with equivalent k' values for FGF-1 and FGF-2. Three others, which showed weaker overall retention for the FGF pair, showed much larger retention differences between FGF-1 and FGF-2. The trends in retention order among the stationary phases are very similar to those seen previously with other unrelated proteins. However, retention differences between the two FGFs, and between the FGFs and lysozyme, do not correlate well with simple charge properties such as net charge, indicating, as in some previous studies, the importance of local regions on the protein surface in determining retention. These observations are interpreted in terms of the structural features of the proteins and the physicochemical properties of the adsorbents.

Keywords: Growth factors; Stationary phases, LC; Proteins; Heparin

1. Introduction

Chromatographic stationary phases from different manufacturers may vary significantly in a number of chemical and physical properties, even when intended for use in similar applications. For the ion-exchange chromatography of proteins, which is examined here, many preparative adsorbent media options are available and in common use. These materials are prepared by different proprietary processes, which may utilize inorganic, organic synthetic and carbohydrate matrices, as well as a variety of spacer-arm and coupling chemistries, to produce numerous combinations of different chemical and physical structures and properties. The individual and cumulative effects of these properties on fundamental chromatographic performance parameters, such as protein retention, are not well understood. As a consequence, the selection of an ion-exchange material that is optimal for a given separation generally proceeds through the experimental screening of a panel of ostensibly similar products that may be quite different in separation performance [1]. Obtaining a better understanding of the relationship between the physicochemical properties of commonly used preparative ion-exchange products, and chromatographic protein retention, offers the potential to guide the selection process, and aid in adsorbent design.

In previous studies [2–4], we have characterized a diverse set of cation-exchange adsorbents by inverse size-exclusion chromatography to determine physical properties such as pore size distribution (PSD) and phase ratios, and compared adsorbent retentivity using three test proteins (lysozyme, cytochrome *c*, α-chymotrypsinogen A). The isocratic retention of each of these proteins was found to vary over several orders of magnitude on the different adsorbents, so that retention and selectivity differences could be correlated

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with the physicochemical properties of the adsorbents. Strong protein retention was found to correlate with adsorbent properties such as PSD, anion type, and spacer-arm length. Consistency in both the magnitude and order of protein retention was observed for the adsorbents and proteins tested, so that differences in protein retentivity among adsorbents were found to be similar for all three test proteins. No selectivity differences were found among the adsorbents, with the order of protein retention always being lysozyme > cytochrome *c* > α-chymotrypsinogen A. The lack of selectivity differences is not surprising, as the proteins used were distinct in sequence and structure, and comprise a set of proteins that are easily separated by cation-exchange chromatography.

The present work utilizes the strong cation-exchangers in the previously characterized adsorbent set, but on proteins that are specifically chosen to reflect selectivity differences. Specifically, we compare the relative retention of two heparin-binding proteins, acidic (aFGF, FGF-1) and basic (bFGF, FGF-2) fibroblast growth factors (FGFs), which have high sequence homology, similar three-dimensional structures and heparin binding sites [5,6]. However, significant differences exist that may be relevant to the retention of these proteins in cation-exchange chromatography, including the number of basic amino acids, and consequently the isoelectric point (p*I*) and the protein net charge at a given pH value.

Comparing the retention of FGF-1 and FGF-2 on a set of cation-exchange adsorbents provides a basis for examining the effect of these protein properties on chromatographic retention. Of particular interest here is the ability of the adsorbent set to discriminate between two proteins of similar mass and structure, but with significant differences in the number of complementary (positive) charges, and the distribution of these charges on the protein surface.

1.1. Charge and structural features of FGF-1, FGF-2 and heparin

FGFs comprise a family of mitogenic and angiogenic heparin-binding proteins that are involved in a variety of cell differentiation and development processes [7]. FGF-1 and FGF-2 both activate signal transduction pathways by binding to the heparin-like carbohydrates of proteoglycans and to tyrosine kinase receptors [8,9]. FGF-1 and FGF-2 have ∼ 55% sequence homology and nearly equivalent molecular weight [10], as shown in Fig. 1 and Table 1. Crystallographic studies have determined both protein structures to consist of 12 antiparallel β-strands, with six of these forming a β barrel [5,11].

The binding of FGFs to heparin has been characterized by a variety of techniques, including isothermal titration calorimetry [12], site-directed mutagenesis [13,14], NMR [6] and X-ray crystallography [15,16]. These studies have defined the structural basis for the binding of heparin fragments to FGFs; a summary of the specific amino acid and carbohydrate interactions for FGF-2 [15] is given in Table 2 and underlined in Fig. 1. In the three-dimensional protein structure, these sequences are spatially adjacent [6,17] and comprise the heparin binding site. The binding sites for FGF-1 and FGF-2 are functionally equivalent in sequence and structure, and are considered to bind heparin in a similar manner [6].

```
                        10         20         30         40
FGF1  FNLP----------PGNYKK PKLLYCSNGG HFLRILPDGT VDGTRDRSDQ

FGF2  PALPEDGGSG AFPPGHFKD PKRLYCKNGG FFLRIHPDGR VDGVREKSDP
             10         20         30         40         50

             50         60         70         80         90
FGF1  HIQLQLSAES VGEVYIKSTE TGQYLAMDTD GLLYGSQTPN EECLFLERLE

FGF2  HIKLQLQAEE RGVVSIKGVC ANRYLAMKED GRLLASKCVT DECFFFERLE
             60         70         80         90         100

             100        110        120        130        140
FGF1  ENHYNTYISK KHAEKNWFVG LKKNGSCKRG PRTHYGQKAI LFLPLPVSSD

FGF2  SNNYNTYRSR KYT--SWYVA LKRTGQYKLG SKTGPGQKAI LFLPMSAKS
             110        118        128        138
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Fig. 1. Alignment of the amino acid sequences of human FGF-1 and FGF-2. Acidic and basic amino acids are shown in bold type. The heparin binding sequences are underlined.

Table 1
Summary of the protein size charge and charge properties

	FGF-1	FGF-2	Lysozyme
Amino acids in sequence	140	146	129
Molecular mass (Da)	15 800	16 400	14 300
Calculated p*I*[a]	7.88	9.59	9.32
Basic amino acids			
Arginine	6	11	11
Lysine	11	14	6
Histidine	5	3	1
Acidic amino acids			
Aspartic acid	7	7	7
Glutamic acid	9	8	2
Net charge at pH 7[b]	+1	+10	+8

[a] Calculated isoelectric points (p*I*) values from the Expert Protein Analysis System (ExPASy) server of the Swiss Institute for Bioinformatics (SIB).
[b] His residues assumed to be deprotonated.

Table 2
Polar contacts between FGF-1 or FGF-2 and the heparin carbohydrates

Heparin contact	Sequence number and amino acid			
	FGF-1		FGF-2	
GlcN-2-*N*-SO_3^- GlcN3-OH	17	S	27	K
GlcN-2-*N*-SO_3^- Idu3-OH	18	N	28	N
GlcN-2-*N*-SO_3^-	92	N	102	N
Idu3-OH	113	K	121	R
GlcN-2-*N*-SO_3^-	118	K	126	K
Idu2-*O*-SO_3^-	128	Q	135	Q
Idu2-*O*-SO_3^- Idu6-CO_2^-	129	K	136	K

The heparin polysaccharide structure is heterogeneous in length and degree of sulfation, but the basic chemical feature is a repeating disaccharide unit of 2-sulfated L-iduronic acid and *N*,6-sulfated D-glucosamine joined by α (1,4) linkages [18,19]. The three-dimensional structure of heparin in solution has been described as a ribbonlike polymer, with sulfate and carboxyl groups projecting outward from the edges [15]. The solution affinity of FGF to heparin was found to be size dependent [20], with a heparin pentasaccharide (FGF-1) [21] or hexasaccharide (FGF-2) [6] considered the minimal sequence for high affinity binding.

Studies with desulfated heparin have concluded that the strong FGF–heparin interaction is electrostatic in origin [22], occurring via complementary charge interactions between the basic binding site and highly sulfated heparin. This interaction is more complex than a simple coulombic interaction; it is estimated that only two to three net ionic interactions are involved, with ∼ 70% of the binding energy resulting from nonionic interactions, with van der Waals interaction and hydrogen bonding important in overall binding strength [12].

Although FGF-1 and FGF-2 contain functionally equivalent heparin binding sites of high homology, significant differences exist in the number of basic amino acids, which is reflected in the very different p*I* values and especially the difference in net charge at pH 7 (Table 1). FGF-1 contains 17 basic residues (lysine + arginine), five of which are located within the heparin binding site, with 12 in the remaining amino acid sequence. FGF-2 contains 25 basic residues, with four in the heparin binding site and 21 in the remaining sequence. FGF-1 contains 16 acidic residues (glutamic + aspartic acid) for a net charge of +1 at pH 7, with FGF-2 containing 15 acidic residues and having a net charge of +10 at pH 7 (these values assume that histidine residues are deprotonated at pH 7).

2. Materials and methods

2.1. Chromatographic stationary phases

Six strong cation-exchangers (SCX) and one immobilized heparin affinity adsorbent were used for this study. TosoHaas SP-550 C and SP-650 M materials were purchased from Tosoh Biosep (Montgomeryville, PA, USA). A "tentacle" type adsorbent, EMD SO_3^- M, was purchased from EM Industries (US associate of E. Merck, Darmstadt, Germany). SP Spherodex was purchased from BioSepra (Marlborough, MA, USA). SP Sepharose Fast Flow was purchased from Amersham Biosciences (Piscataway, NJ, USA). Cellufine Sulfate was purchased from Amicon (Beverly, MA, USA). Heparin Superflow Plus heparin affinity adsorbent was purchased from Sterogene Bioseparations (Arcadia, CA, USA).

The SCX adsorbents selected differ in particle morphology, the chemical nature of the base matrix, the spacer-arm chemistry, the anionic ligand, and the ligand density. The physicochemical properties of these adsorbents, including particle diameter, the chemical nature of the base matrix, spacer-arm length and chemical structure, anionic ligand density, and pore size distribution determined by inverse size exclusion chromatography have been summarized previously [2,3] and are not given here. The SP nomenclature used for SP Spherodex and SP Sepharose FF has in these cases been applied to structures other than sulfopropyl groups, with the SP Spherodex using a sulfate group attached directly to a dextran polymer, and the SP Sepharose FF utilizing a 3-(2-hydroxypropoxy)-1-propane sulfonic acid moiety.

2.2. Protein samples and preparation

FGF-1 and FGF-2 were purified from *Escherichia coli* fermentations by a combination of cation-exchange, heparin affinity, and hydrophobic interaction chromatography [23]. FGF solutions were prepared by dialysis into 10 mM sodium phosphate at pH 7, and then filtered through Millipore Millex-GV 0.22 micron filters (Bedford, MA, USA). After filtration, FGF solutions were diluted with the 10 mM sodium phosphate to obtain a final concentration of 4 mg/ml.

2.3. Instrumentation and methods

The columns, adsorbent preparation, packing procedures, chromatography system and methods used to obtain isocratic k' values for FGF-1 and FGF-2 were essentially as described previously [3]. For this application, mobile phase A contained 10 mM sodium phosphate buffer at pH 7, and mobile phase B contained 2 M NaCl in 10 mM sodium phosphate at pH 7. Isocratic elution of proteins with high k' values results in broad peaks, and the values of t_R reported here are based on the peak maxima. Retention times at each NaCl concentration were the average of duplicate injections.

Heparin affinity chromatography was carried out using the columns, packing procedures and flow rates described for the SCX media, and the mobile phases given above. A linear gradient from 0 to 100% mobile phase B was carried out over 12 bed volumes, with FGF-1 and FGF-2 chromatographed both separately and as a co-mixture of equal masses of each.

Retention differences that arise from adsorbent phase ratio differences, and not intrinsic protein–stationary phase equilibrium, are normalized across this adsorbent set by dividing k' values by the respective adsorbent phase ratios, as previously described [3]. With this approach, adsorbent phase ratio values obtained for a dextran solute with a viscosity radius of 1.77 nm were used [2], and the resulting normalized retention is referred to here as *K*.

3. Results and discussion

3.1. Chromatographic retention of FGF-1 and FGF-2

An initial comparison of the retention of FGF-1 and FGF-2 on an immobilized heparin affinity stationary phase

Table 3
Concentration of NaCl at the peak maxima for the elution of FGF-1 and FGF-2 from the heparin affinity column

FGF type	NaCl (M) at peak maxima
FGF-1	1.51
FGF-2	1.67

was made to determine the effect of sequence differences on heparin binding. Isocratic elution of the FGFs was characterized by extreme peak tailing (data not shown), so that this baseline comparison was carried out with a linear NaCl gradient, with the NaCl concentration at the peak maxima used to compare retention.

The relative retention of the FGFs (Table 3) shows FGF-2 retention to be measurably greater than that of FGF-1, although the difference is not sufficient to permit separation when co-mixed in a 1:1 mass ratio (data not shown). This comparison demonstrates that chromatographic interaction with the heparin binding site results in essentially equivalent retention for FGF-1 and FGF-2.

The isocratic retention of FGF-1 and FGF-2 on the SCX adsorbents, shown as log–log plots of k' versus [NaCl], is given in Fig. 2a and b. These results reflect contributions due to the structures of both the proteins and stationary phases involved, as examined previously for our more disparate set of model proteins [3]. The discussion below of the FGF results is organized to facilitate further insight into these structural contributions via two retention comparisons. In the first (Section 3.2), the retention of either FGF-1 or FGF-2 is compared separately across the adsorbent set, while in the second (Section 3.3) the retention of FGF-1 is compared to that of FGF-2 on each adsorbent to provide an indication of selectivity patterns.

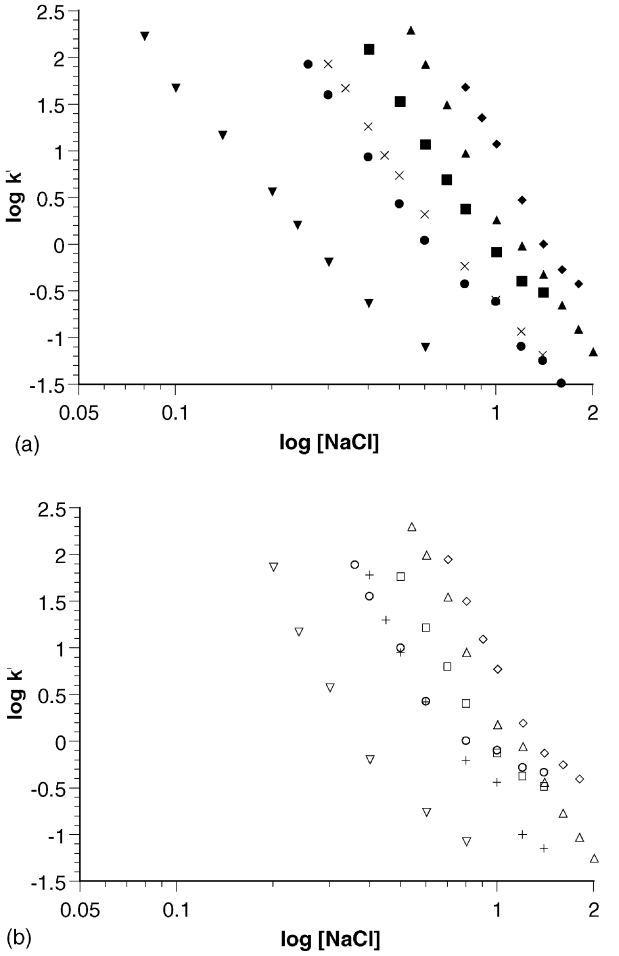

Fig. 2. (a) Log k' vs. log [NaCl] for FGF-1 on SP Spherodex M (◆), Cellufine Sulfate (▲), EMD EM SO_3^- M (■), TosoHaas SP-550 C (×), SP Sepharose FF (●) and SP-650 M (▼). (b) Log k' vs. log [NaCl] for FGF-2 on SP Spherodex M (◇), Cellufine Sulfate (△), EMD EM SO_3^- M (□), TosoHaas SP-550 C (+), SP Sepharose FF (○) and SP-650 M (▽).

3.2. FGF-1 or FGF-2 retention across the adsorbent set

The comparison of the isocratic retention of either FGF-1 or FGF-2 across the adsorbent set examines the extent to which differences in the physical and chemical properties of these adsorbents affect chromatographic retention. To compare more accurately isocratic k' values obtained on adsorbents of differing porosity, the k' values are normalized by dividing the experimentally determined k' values by the adsorbent phase ratio to correct for surface area differences among adsorbents [2,3]. The resulting normalized retention, K, in units of nanometers, is plotted in log–log format against NaCl concentration in Fig. 3a and b. Cellufine Sulfate has pore dimensions that should exclude most protein-sized solutes, and the k' values are, therefore, not normalized, as this material would represent the adsorbent of minimum surface area per unit volume.

In agreement with what was found for lysozyme, cytochrome *c* and α-chymotrypsinogen A [3], the retention plots for each FGF on the different adsorbents in Figs. 2 and 3a or 2 and 3b are largely parallel, with similar slope values and little crossover. At high [NaCl] (>1 M), deviations in linearity are observed for some of the FGF-2 plots; it has been argued that at such high salt concentrations the potential for the addition of hydrophobic and other non-electrostatic interactions often results in curvature of these plots on ion-exchange media [24].

Non-mechanistic models for protein ion-exchange chromatography, such as the stoichiometric displacement model (SDM) [25], have interpreted the slope of such log–log plots to be indicative of the number of discrete coulombic interactions between the protein and the stationary phase. The slope value, Z, can then be used to compare quantitatively retention differences seen for different proteins on the same stationary phase [26–28]. Here, although slope differences between FGF-1 and FGF-2 on a given stationary phase were observed (Section 3.3), the parallel nature of these plots for the same protein on different adsorbents makes the Z value much less useful as a means to compare adsorbent retention.

Table 4
The normalized retention (K) values for FGF-1 and FGF-2 for five cation-exchange adsorbents

[NaCl] (M)	Normalized retention K (nm)									
	FGF-1					FGF-2				
	SP Spherodex M	EMD SO_3^- M	SP-550 C	SP Sepharose FF	SP-650 M	SP Spherodex M	EMD SO_3^- M	SP-550 C	SP Sepharose FF	SP-650 M
1.40										
1.20	5					2.5				
1.00	19					10				
0.90	37					20				
0.80	78	4.4				51	4.6			
0.70		9					11			
0.60		22	3.3	2.5			30	4.1	6	
0.50		62	9	6			105	14	23	
0.45			14					31		
0.40			28	20	1			94	82	3
0.36				32					178	
0.30					3					17
0.24					7					67

To facilitate a more quantitative comparison of retention differences, four normalized K values for both FGFs on each adsorbent are compared in Table 4. These values were obtained at the lowest 4 NaCl concentrations common to both the FGF-1 and FGF-2 datasets on each adsorbent. Adsorbent differences may then be compared by an ordering of K values at equal NaCl concentrations.

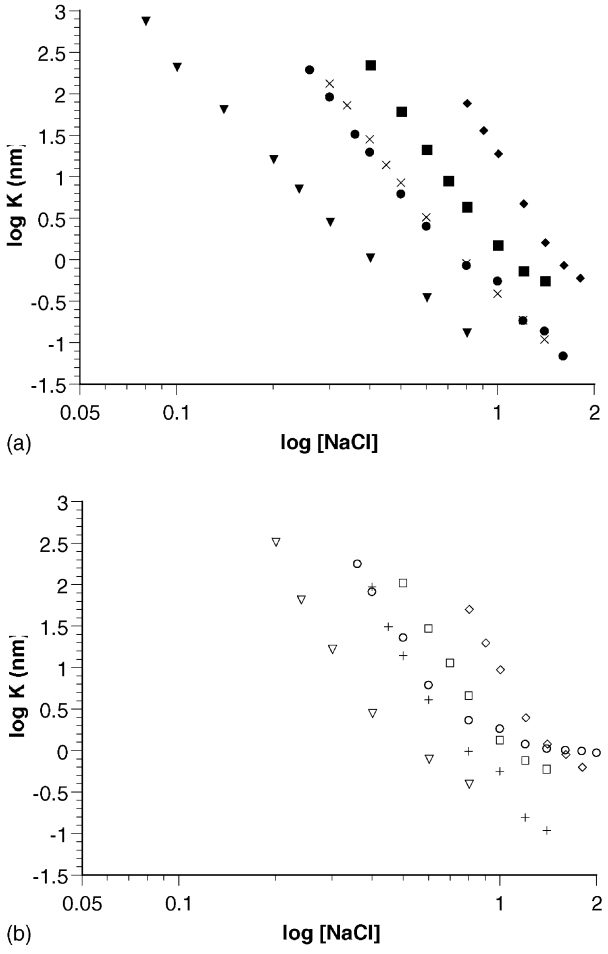

Fig. 3. Log K vs. log [NaCl] for (a) FGF-1 (b) FGF-2. Legends as in Fig. 2a and b, respectively.

The normalized retention data of FGF-1 and FGF-2 in Fig. 3a and b are in good agreement with several findings from our previous retention comparison using less structurally related proteins [3]. First, for both FGFs, very large and significant rentention differences are seen across the adsorbent set, with k' values measured at any given NaCl concentration found to span a range of several orders of magnitude. Correction for surface area differences does not reduce the magnitude of the retention differences among the adsorbents in this set, as shown by the comparison of Figs. 2a,b and 3a,b. As in the previous work, this confirms that protein retention differences are caused primarily by physicochemical differences among the adsorbents, and not by phase ratio differences.

Understanding the adsorbent and protein properties that give rise to these retention differences is clearly complex, as this involves the comparison of materials that vary simultaneously in more than one chemical or physical property. The multivariate nature of the problem means that some uncertainty is inherent in any interpretation of retention differences; the approach here assumes that log scale retention differences are not generally indicative of subtle properties, so that trends and correlations between retention data and adsorbent properties can be identified. In our previous work [3], adsorbent ionic capacity and surface charge density, as well as adsorbent hydrophobicity, did not correlate well with protein retention, but a good correlation between adsorbent PSD and retention was found. In particular, the adsorbents with significant pore volume of dimensions approximately equal to those of the protein solute (SP Spherodex M, EMD SO_3^- M, SP-550 C), where ligand groups could be envisioned to reach extended regions of the surfaces of the protein solutes or binding could occur at more than one surface of the protein, displayed relatively strong protein retention.

Conversely, retention was relatively weak on those adsorbents with significant pore volume of dimensions much greater than those of the protein solute (SP Sepharose FF, SP-650 M). Cellufine Sulfate, although marginally permeable for protein sized solutes, was proposed to function in an analogous manner, with limited accessible pore volume and a rugose surface providing the surrounding charge.

The relative retention order of FGFs on this adsorbent set follows the trend described previously, so that the underlying correlations described above would be expected to apply here. The strongest FGF retention was found on SP Spherodex, Cellufine Sulfate and EMD SO_3^- M, the weakest retention on SP Sepharose FF and SP-650 M, with the SP-550 C retention falling in between, as seen in Fig. 3a and b. In the FGF dataset, the potential for affinity type interactions between FGFs and SP Spherodex M and Cellufine Sulfate, which is described in more detail below, means that heparin mimicry, and not just a PSD effect, may contribute to the strong retention observed. A comparison of the normalized retention of either FGF on the two TosoHaas adsorbents shows the SP-550 C and SP-650 M to display the effect of the adsorbent pore size distribution. In this comparison, in which the adsorbent base matrix and anionic charge group chemistry are equivalent, and surface area differences are normalized, the retention of both FGFs is much greater on the SP-550 C than the SP-650 M. Adsorbent flexibility may also be a factor in the strong adsorption on the three most retentive materials, but the relatively strong retention on the inflexible SP-550 C places this possibility in question.

3.3. Relative retention of FGF-1 and FGF-2 on each SCX adsorbent

The second comparison examines the relative retention of FGF-1 and FGF-2 on each adsorbent, as shown in Fig. 4a-f. With these plots, selectivity is examined through the ability of the adsorbent to discriminate chromatographically between the two FGFs. The isocratic retention of lysozyme on this same adsorbent set [3] is added to these FGF plots to assess adsorbent selectivity further. In these comparisons, the k' values for FGF-1, FGF-2 and lysozyme are considered in relation to each other, and not to values on other adsorbents, so that normalization is not required. This allows Cellufine Sulfate, for which a phase ratio and lysozyme isocratic k' values could not be determined, to be included in the dataset. The k' values at the same four common NaCl concentrations used for normalized K values are given in Table 5, along with the available corresponding values for lysozyme.

The retention of FGF-1 is slightly greater than that of FGF-2 on SP Spherodex, approximately equivalent on Cellufine Sulfate, and slightly lower than FGF-2 on the EMD SO_3^- M. The differences in k' in these three adsorbents are in most cases quite small (<30%), and in all cases less than a factor of 2. For TosoHaas SP-650 and SP Sepharose FF, the FGF-2 retention was significantly greater (3–17-fold) than that of FGF-1 across the full range of NaCl concentrations examined. SP-550 C showed a more complex retention relationship, with FGF-2 retention significantly greater at lower [NaCl] and higher k' values, but equivalent at higher [NaCl], where k' values were much lower. A general qualitative correlation is apparent from these data: the adsorbents displaying the strongest overall retention for FGFs (SP Spherodex, Cellufine Sulfate, EMD SO_3^- M) show weak selectivity between FGF types, while those adsorbents with weaker retention (SP-550 C, SP Sepharose FF, SP-650 M) show greater selectivity.

The comparison of lysozyme retention to that of FGF for the five adsorbents for which data are available shows that for three of them, SP Spherodex M, EMD SO_3^- M and SP Sepharose FF, the k' values for lysozyme are approximately 10-100 fold lower than for the FGFs. This is in contrast to the TosoHaas materials, where retention differences between lysozyme and the FGFs are much smaller, with the retention of lysozyme greater than that of FGF-1 on SP-650 M. Thus FGF versus lysozyme selectivity is correlated with strong FGF retention, although SP Sepharose FF presents some uncertainty in this analysis.

The retention of the three proteins does not correlate with simple charge effects; for example, on only one adsorbent, SP-650 M, was protein retention increasing with net positive charge, where FGF-2 at +10 > Lys at +8 > FGF-1 at +1. A better correlation is seen with the number of basic residues, which predicts FGF-2 > FGF-1 > lysozyme (Table 1), but this correlation is inconsistent with our earlier data on other proteins [3]. Thus further consideration of protein and adsorbent charge and structural features is necessary.

In the three-dimensional structure of the FGFs, represented by FGF-1 in Fig. 5 [29], the heparin binding site, seen as a cleft, and the surrounding surface contain seven basic residues in close proximity. This area lacks acidic residues, and therefore, represents a region of high positive charge density. Such regions of high charge density, complementary to that of the stationary phase, have been referred to as chromatographic contact regions [30] or ionotypes [31]; the latter term denotes the discrete and stoichiometric nature of the adsorbent protein interaction postulated by the SDM, but the concept of energetically favored association between surfaces of high charge density applies outside the context of this model as well. A comparable region of high localized positive charge density is not observed in the lysozyme structure, which shows a more uniform charge distribution. Thus FGF may be structurally configured for multiple coulombic interactions with the adsorbents to a greater degree than lysozyme, potentially giving rise to stronger retention on cation-exchange materials.

This protein structural feature does not, however, translate to significantly greater chromatographic retention of FGFs on all of the adsorbents examined here: for TosoHaas SP-550 C the retention of FGF-1 and lysozyme are nearly the same, while on SP-650 M the retention of lysozyme is greater than that of FGF-1. As seen in the previous section,

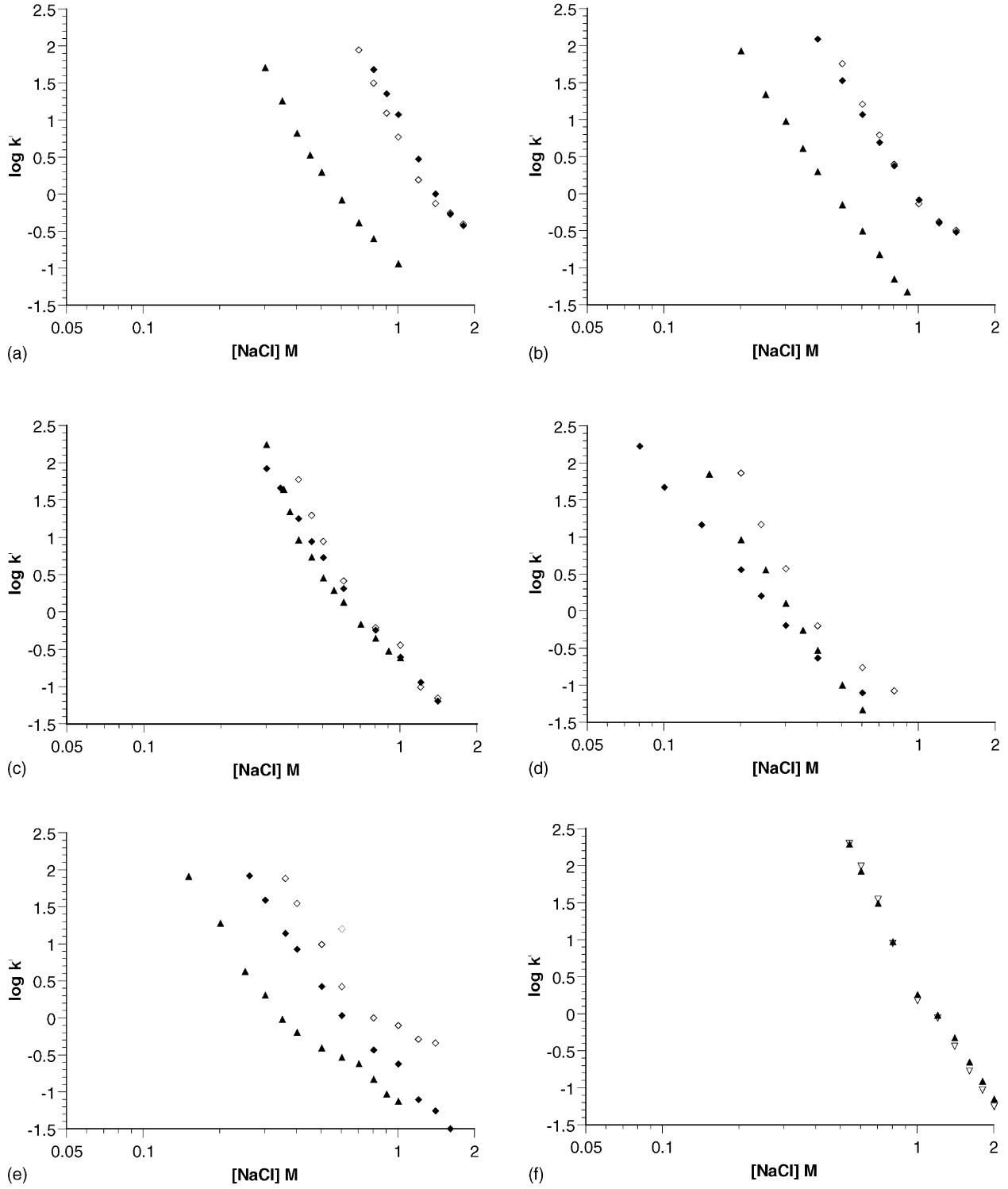

Fig. 4. Log k' vs. log [NaCl] for FGF-1 (◆), FGF-2 (◇) and lysozyme (▲) on (a) BioSepra SP Spherodex M, (b) EMD EM SO_3^- M, (c) TosoHaas SP-550 C, (d) TosoHaas SP-650 M, (e) SP Sepharose FF, (f) Amicon Cellufine Sulfate.

adsorbent properties also play an important role in retention, presumably because the position of the protein relative to the charged adsorbent ligands ultimately determines what interactions are possible. The relative retention of FGF-1 and FGF-2 may reflect such differences in the orientation of the adsorbent–protein interaction. Equivalent FGF retention on an adsorbent would imply that the multiple additional positive charges on the FGF-2 are not participating chromatographically, and that adsorbent interaction is occurring through the positively charged amino acid sequences

Table 5
The k' values for three proteins on six cation-exchange adsorbents, at each of four NaCl concentrations

[NaCl] (M)	k'									[NaCl] (M)	k'									
	SP Spherodex			Cellufine Sulfate			EMD SO_3^- M				SP-550 C			SP Sepharose FF			SP-650 M			
	FGF-1	FGF-2	Lys	FGF-1	FGF-2	N/A	FGF-1	FGF-2	Lys		FGF-1	FGF-2	Lys	FGF-1	FGF-2	Lys	FGF-1	FGF-2	Lys	
1.40										0.60	2.1	2.6	1.4	1.1	2.7	0.3				
1.20	3	1.6								0.50	5.4	9	3	2.7	10	0.4				
1.00	12	6	0.1							0.45	9	20	6							
0.90	23	13								0.40	18	61	9	9	36	0.7	0.2	0.6	0.3	
0.80	49	32	0.3	9.5	9.1		2.4	2.5	0.1	0.36				14	78	1				
0.70				32	35		5	6	0.2	0.30							0.7	4	1.3	
0.60				86	100		12	16	0.3	0.24							1.6	15		
0.54				199	202					0.20							4	74	9	
0.50							34	58	0.7											

in and around the heparin binding site, which is conserved and functionally equivalent on both FGFs.

This scenario appears a likely one for SP Spherodex, Cellufine Sulfate, and EMD SO_3^- M, all of which show the very strong FGF retention that would be expected for this pseudo FGF–heparin binding. SP Spherodex and Cellufine Sulfate are both sulfated carbohydrates; the latter adsorbent is often used as a heparin affinity substitute, so the potential to mimic an FGF–heparin interaction, with perhaps a less specific steric fit, is not surprising. EMD SO_3^- M is a synthetic polymer, with monomeric subunits that are structurally quite different from the heparin carbohydrates [32], so that such recognition is less expected, but may occur as a more general interaction between patterned matched regions of opposite charge on the protein and adsorbent. This possibility is supported by several related studies:

(1) Studies examining the interaction of FGF-1 with non-heparin polyanions have found that FGF-1 binding is not highly specific toward the structure of the polymers tested, and occurs with a diverse set of anionic biopolymers, including sulfated carbohydrates, nucleic acids, homopolymers of acidic amino acids and phosphoproteins [33,34]. These polymers are structurally diverse, but all contain linear regions of repeating negative charge.

(2) A comparison of the structural features in heparin-binding proteins suggests a common spatial motif in heparin-binding proteins [35]. For a variety of heparin-binding proteins examined, FGF-2 among them, the heparin binding sites contained basic amino acids, with two of these at the edges of a pocket. The separation of these two basic residues was found to be $\sim$ 20 Å for helical structures, and $\sim$23 Å for beta sheet structures, with the charges facing opposite sides in both cases.

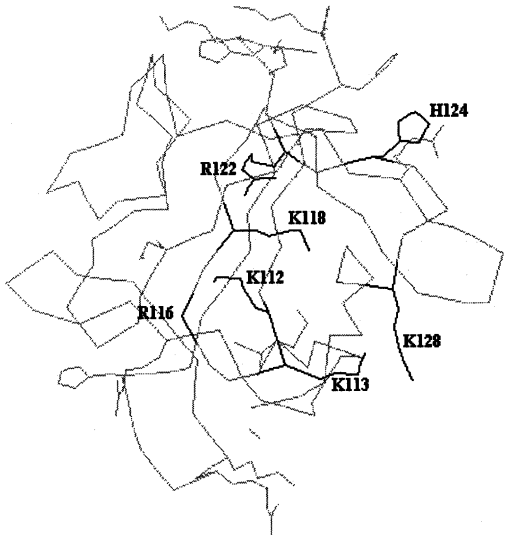

Fig. 5. Structure of FGF-1, highlighting the basic residues in and around the heparin binding. (http://www.expasy.ch/spdbv/ Chain 1BAR:A).

This spacing was found to correspond to charges on opposite ends of a heparin pentasaccharide accommodated in the binding site, perhaps with intertwining of the heparin–protein complex. Such intertwining is not suggested here for the protein–adsorbent interaction, but the regular spacing of sulfate groups on long carbohydrates or even the synthetic EMD SO_3^- M polymer may permit some interaction with the heparin binding site. These linear polyanions may have the charge spacing necessary to form the two or three critical ionic interactions with the FGF heparin binding site, although with a less optimal steric fit than occurs for heparin and, therefore, less specificity for the non-electrostatic interactions.

(3) The heparin binding site is located in a structurally flexible region [7], so that the side chains have some ability to adapt their location to attain charge complementarity. Flexibility of the stationary phase may contribute as well, with the highly retentive SP Spherodex, Cellufine Sulfate, and EMD SO_3^- M all expected to display local flexibility.

For SP Sepharose FF, SP-650 M, and SP-550 C, FGF-2 retention is significantly greater than FGF-1, but the retention of both is less than for the other adsorbents tested. These materials could also display a heparin-like interaction with the heparin binding site, but the retention behavior shows that specific structural characteristics of these materials attenuate its dominant role, with at least some of the additional basic charges on the FGF-2 contributing to, and increasing, chromatographic retention relative to FGF-1. The anionic groups in these adsorbents are located on the ends of alkyl spacer-arms derivatized onto neutral polymers, and differences in both the base matrices and the alkyl chains may contribute to the performance differences. SP Sepharose FF appears to represent the extreme case of a spacer-arm effect, with the anionic group on the end of a seven-atom chain projecting outward from the carbohydrate surface [2], so that making complementary charge interactions at the ends of the binding site while simultaneously accommodating the linear polysaccharide within the site may be sterically more difficult. An additional factor may be that the agarose forming the base matrix comprises bundles of agarose fibers that impart greater rigidity, thereby making it less able to adapt to provide good charge complementarity with the basic groups on the proteins than is possible for the Spherodex and Cellufine base matrices and the EM tentacles. The TosoHaas materials, on methacrylate base matrices, are intrinsically more rigid and employ propyl spacer arms, which again can inhibit extensive geometric conformation and charge pairing.

Therefore, our results are consistent with the hypothesis that equivalent and strong retention of both FGFs occurs through adsorbent–protein interaction at the heparin binding site. Steric preclusion by spacer-arm length, matrix rigidity and/or lack of regular charge spacing from extensive physical contact and strong interaction with the heparin binding site would mean that chromatographic retention occurs through interactions in the remainder of the protein sequence. This contact with additional surface charge outside the heparin binding site is reflected in the decreased overall retention of both FGFs, but the increased selectivity of FGF-2 relative to FGF-1.

4. Conclusions

Significant retention and selectivity differences were observed among the cation-exchange adsorbents examined with FGF-1, FGF-2 and lysozyme. FGF-1 and FGF-2 are similar in mass, structure, and heparin binding sites, yet display significant differences in such global properties as protein p*I*, number of basic charges, and net charge; they therefore provide a useful solute pair for examining the molecular basis for protein retention and selectivity.

The comparative retention results of the different proteins on the diverse set of stationary phases provide insights into the origins of both strong retention and high selectivity, which are not necessarily correlated. Retention in cation-exchange chromatography appears to be strengthened primarily by the clustering of positive charges over an extended region of the molecular surface that is substantially devoid of negative charges; the converse would presumably hold in anion-exchange chromatography. Close interaction with such a “patch” appears to be promoted by a stationary phase on a flexible matrix. To the extent the resulting retention is stronger than that of any other protein in the feed, this strong retention can yield high selectivity, but in the presence of structurally similar molecules selectivity is likely to be low. This is the case for the FGFs on the high-retention adsorbents in this work, but may also be true for variants present in proteins produced by rDNA technology. For situations such as this, adsorbents on which specific interactions are suppressed and interactions with a larger fraction of the adsorbent surface are manifested may be more suitable for attaining high selectivity, albeit at lower retention levels. Such behavior appears to be favored by more rigid matrices, with low ligand densities also potentially helping to suppress the tendency for adsorption to occur predominantly through a localized region on the protein surface.

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