Energetic Characterization of the Basic Fibroblast Growth Factor–Heparin Interaction: Identification of the Heparin Binding Domain†

Leo D. Thompson,‡ Michael W. Pantoliano, and Barry A. Springer*

Crystallography and Biophysical Chemistry, The DuPont Merck Pharmaceutical Company, DuPont Experimental Station, P.O. Box 80228, Wilmington, Delaware 19880-0228

Received November 12, 1993; Revised Manuscript Received January 13, 1994®

ABSTRACT: Fibroblast growth factors (FGF's) interact on cell surfaces with "low-affinity" heparan sulfate proteoglycans (HSPG) and "high-affinity" FGF receptors (FGFR) to initiate cell proliferation. Previous reports have implicated the binding of heparin, or heparan sulfate, to FGF as essential for FGF-mediated signal transduction and mitogenicity. However, the molecular recognition events which dictate the specificity of this interaction have remained elusive. Amino acid residues on the surface of basic FGF (bFGF) were targeted as potential heparin contacts on the basis of the position of sulfate anions in the X-ray crystal structure of bFGF and of a modeled pentasaccharide heparin–bFGF complex. Each identified amino acid was replaced individually with alanine by site-directed mutagenesis, and the resulting mutant proteins were characterized for differences in binding to a low molecular weight heparin (~3000) by isothermal titrating calorimetry and also for differences in [NaCl] elution from a heparin–Sepharose affinity resin. The combination of site-directed mutagenesis and titrating calorimetry permitted an analysis of the energetic contributions of individual bFGF residues in the binding of heparin to bFGF. The key amino acids which comprise the heparin binding domain on bFGF constitute a discontinuous binding epitope and include K26, N27, R81, K119, R120, T121, Q123, K125, K129, Q134, and K135. Addition of the observed $\Delta\Delta G°$ of binding for each single site mutant accounts for 8.56 kcal/mol (>95%) of the free energy of binding. The $\Delta\Delta G°$ values for N27A, R120A, K125A, and Q134A are all greater than 1 kcal/mol each, and these four amino acids together contribute 4.8 kcal/mol (56%) to the total binding free energy. Amino acid residues K119 through K135 reside in the C-terminal domain of bFGF and collectively contribute 6.6 kcal/mol (76%) of the binding free energy. Although 7 out of the 11 identified amino acids in the heparin binding domain are positively charged, a 7-fold increase in [NaCl] decreases the affinity of wild-type bFGF binding to heparin only 37-fold (K_d at 0.1 M NaCl = 470 nM vs K_d at 0.7 M NaCl = 17.2 μM). This indicates that pure electrostatic interactions contribute only 30% of the binding free energy as analyzed by polyelectrolyte theory and that more specific nonionic interactions, such as hydrogen bonding and van der Waals packing, contribute the majority of the free energy for this binding reaction.

Basic fibroblast growth factor (bFGF)[1] and acidic FGF (aFGF) are potent mitogenic polypeptides that modulate cell proliferation and differentiation and promote wound repair and angiogenesis (Burgess & Maciag, 1989; Davidson et al., 1985; Gospodarowicz, 1987; McGee et al., 1988; Nabel et al., 1993; O'Keefe et al., 1988; Shipley et al., 1989). As mediators of angiogenesis, bFGF and aFGF also have been implicated in a number of diseases including the growth of solid tumors, diabetic retinopathy, and rheumatoid arthritis (Folkman & Klagsbrun, 1987; Moses & Langer, 1991). Basic FGF is one member of nine currently identified FGF's that share amino acid sequence homology and the ability to control cell growth and exhibit a high specificity for the sulfated polysaccharides heparin and heparan sulfate. The ability to bind heparin has permitted facile purification of bFGF (Shing et al., 1984; Gospodarowicz et al., 1984; Klagsbrun & Shing, 1985) and has been identified as a requirement for bFGF-dependent biological activity (Rapraeger et al., 1991; Yayon et al., 1991).

At least two distinct receptor classes on the cell surface are known to interact with bFGF. The first, commonly referred to as the "low-affinity" receptor (IC_{50} ~ 5–50 nM), has been identified as a heparan sulfate proteoglycan (HSPG) (Vlodavsky et al., 1987; Moscatelli, 1988; Bashkin et al., 1989). The HSPG's are a polymorphic group of polypeptide cell surface receptors laden with sulfated polysaccharides on the extracellular domains. The intracellular domains of HSPG's interact with actin (Saunders et al., 1989). The second receptor that binds FGF is one of at least four currently identified members of immunoglobulin-like FGF receptors. Designated as FGFR1, -2, -3, or -4, the FGFR's have intracellular tyrosine kinase domains and have been termed "high-affinity" receptors (IC_{50} ~ 10–200 pM) (Moscatelli, 1987; Neufeld & Gospodarowicz, 1985). It has been reported that on cell surfaces bFGF must bind first to the HSPG receptor before binding to the FGFR and subsequent mitogenesis can occur (Rapraeger et al., 1991; Yayon et al., 1991). Similar results have been reported for bFGF binding to a soluble FGFR (Ornitz et al., 1992). More recent measurements of FGF binding to a soluble FGFR in the absence of heparin, however, refute this latter suggestion (Kiefer et al., 1992; Pantoliano et al., 1994).

† Supported by the DuPont Merck Pharmaceutical Co.

* To whom correspondence should be addressed.

‡ Present address: GlycoTech Corp., Rockville, MD 20850.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; HSPG, heparan sulfate proteoglycan; FGFR, fibroblast growth factor receptor; aFGF, acidic fibroblast growth factor; H.Seph., heparin–Sepharose; low MW, low molecular weight; DTT, dithiothreitol; SD, standard deviation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SOS, sucrose octasulfate; MPI, mucus proteinase inhibitor. Single-letter amino acid code is used throughout. bFGF mutations are designated by the one-letter code for the target residue, the residue number [based on the bFGF sequence numbering of Zhang et al. (1991)], and the one-letter code for the resultant residue change.

0006-2960/94/0433-3831$04.50/0

Heparin binding to FGF also has been shown to impart protection from proteolytic, thermal, and pH-dependent degradation and denaturation (Gospodarowicz & Cheng, 1986; Saksela et al., 1988; Sommer & Rifkin, 1989). Therefore, heparin binding may provide a storage and regulated release mechanism for FGF from cell surfaces (Flaumenhaft et al., 1990); however, it is not clear what role HSPG, either cell-associated or free in the extracellular matrix, plays in initiating mitogenesis.

Identification and characterization of the FGF heparin binding site have been attempted using a variety of techniques, including reductive methylation of aFGF lysine residues (Harper & Lobb, 1988), bFGF deletion mutation analysis (Seno et al., 1990), synthetic peptide studies (Baird et al., 1988), X-ray crystallography (Ago et al., 1991; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1993), and site-directed mutagenesis (Burgess et al., 1990; Heath et al., 1991; Presta et al., 1992). These efforts suggested and confirmed several residues which interact specifically with heparin; however, a complete identification and energetic characterization of the heparin binding site on FGF had not been reported. On the basis of the published X-ray crystal structures of bFGF with bound sulfate anions (Eriksson et al., 1991; Zhang et al., 1991) and a molecular model of a pentasaccharide heparin analog docked to bFGF (this paper), we were able to target 11 amino acid residues on the protein surface likely to be important for heparin binding. Identified amino acids were individually replaced with alanine by site-directed mutagenesis and characterized by [NaCl]-dependent elution from a heparin–Sepharose (H.Seph.) affinity column and for differences in binding a low molecular weight (MW) heparin by isothermal titrating calorimetry. This work represents a continuation of the energetic characterization of the individual binary reactions of bFGF with heparin, FGFR1 with heparin, and bFGF with FGFR1 (Pantoliano et al., 1994).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Restriction enzymes T4 polynucleotide kinase and T4 DNA ligase were obtained from Gibco/BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and used according to manufacturer's recommendations. Low MW heparin (average M_r = 3000) was obtained from Sigma (St. Louis, MO) and used without further purification. Heparin 5000 (average M_r = 4800) was purchased from Calbiochem (San Diego, CA) and is identical to RD Heparin 5000 from Hepar Industries (Franklin, OH). Hepes and dithiothreitol (DTT) were purchased from Research Organics Inc. (Cleveland, OH). All other chemicals were purchased from either Sigma (St. Louis, MO) or United States Biochemicals (Cleveland, OH).

DNA Manipulations. The bFGF expression plasmid (pT7bFGF) was constructed as described previously (Squires et al., 1988; Pantoliano et al., 1994) and encodes a 157 amino acid form of bFGF. A derivative of pT7bFGF (pBF01) was engineered by introducing restriction enzyme sites for *Eco*RI, *Xho*I, *Apa*I, and *Afl*II via site-directed mutagenesis of pT7bFGF. Introduction of these restriction sites did not alter the amino acid sequence of bFGF. The bFGF fragment from pBF01 was cloned into M13mp19 and used as a source of template DNA for site-directed mutagenesis. Except for the K119A/R120A/K125A/K129A quadruple mutant, all mutations were constructed by single-stranded oligonucleotide-directed mutagenesis using the USB T7-GEN *in vitro* mutagenesis kit (United States Biochemicals, Cleveland, OH) according to manufacture's recommendations. All mutations were confirmed by dideoxy sequence analysis using the Sequenase DNA sequencing kit (United States Biochemicals, Cleveland, OH). Mutant bFGF fragments were subcloned back into pT7bFGF for protein expression. The K119A/R120A/K125A/K129A quadruple mutant was constructed using two overlapping oligonucleotides with the codon encoding alanine (GCG) replacing the codons at amino acid positions 119, 120, 125, and 129 in bFGF. After annealing, the oligonucleotide complex containing 5′ *Xho*I and 3′ *Apa*I compatible ends was cloned into the *Xho*I and *Apa*I sites of pBF01.

Expression and Purification of Wild-Type and Mutant bFGF Proteins. Protein expression from wild-type and mutant bFGF constructs was accomplished in *Escherichia coli* BL21-(DE3) (Novagen, Madison, WI). For each construct a single colony isolate was inoculated into 2× YT media (5 g of NaCl, 10 g of yeast extract, 16 g of BactoTryptone per liter) supplemented with 150 μg/mL carbenicillin and grown to early log phase at 30 °C with shaking. Typically, 1 L of 2× YT media containing 150 μg/mL carbenicillin was inoculated with 10 mL of this starter culture and incubated at 30 °C with shaking until an A_{600nm} = 0.80 was achieved, at which time isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the T7 polymerase gene. Following induction, cultures were allowed to grow for an additional 5 h. The cells were harvested by centrifugation, and the cell paste was stored at −80 °C.

The wild-type and mutant bFGF proteins were purified essentially as previously described (Thompson et al., 1991). Proteins were loaded and eluted from an SP Sephadex C25 column (Pharmacia) followed by a heparin–Sepharose (H.Seph.) HiTrap column (Pharmacia). Due to the nature of the introduced mutations, the NaCl gradients necessary for suitable elution from SP Sephadex and H.Seph. were determined empirically. Purification of the K119A/R120A/K125A/K129A mutant required 25–600 and 100–600 mM NaCl linear gradients for elution from SP Sephadex and H.Seph., respectively. Purification of the K125A mutant required 100–600 and 250–750 mM NaCl gradients, whereas the remaining mutants and wild-type bFGF required 100–600 and 600–2000 mM NaCl gradients for elution. Purified proteins were typically concentrated to 5 mg/mL and exchanged into storage buffer (20 mM sodium phosphate, pH 7.1, 100 mM NaCl, 5 mM EDTA, 1 mM DTT, 10% glycerol, v/v) using an Amicon YM10 centriprep concentrator (Beverly, MA). Samples were stored at −20 °C. All purified proteins were >95% pure as determined by SDS-PAGE analysis.

To ensure that the introduction of single and multiple surface amino acid residue mutations did not perturb the overall bFGF structure, circular dichroism (CD) spectra were obtained for each protein. The CD spectra for all mutants, except the K119A/R120A/K125A/K129A quadruple mutant, were indistinguishable from the wild-type protein, indicating no dramatic perturbations in secondary and tertiary structure (data not shown). The K119A/R120A/K125A/K129A mutant did show minor CD spectral differences compared to wild-type bFGF, which might be consistent with small structural differences for this mutant protein.

[NaCl]-Dependent Elution from Heparin–Sepharose. [NaCl] elution profiles of wild-type and mutant bFGF proteins were obtained by loading 100 μg of purified protein in 500 μL of loading buffer (20 mM sodium phosphate, pH 7.1, 5 mM EDTA, 10 mM DTT, 25 mM NaCl) onto a 1.0-mL H.Seph. HiTrap affinity column (Pharmacia) attached to an FPLC

system (Pharmacia). The H.Seph. column with bound protein was washed with 10 column volumes of loading buffer, and protein was eluted with a linear 100-mL, 25–3025 mM NaCl gradient in loading buffer.

Isothermal Titrating Calorimetry. Protein samples were dialyzed for 16 h against 2 × 1 L changes of titration buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM DTT). The concentration of the protein was determined spectrophotometrically ($A^{0.1\%}_{280nm}$ = 0.964; Pantoliano et al., 1994) and adjusted to the appropriate concentration (between 100 and 200 μM) by dilution with titration buffer. Low MW heparin (average M_r = 3000) was dissolved in the titration buffer at a 5-fold higher concentration relative to the protein. All solutions were passed through a 0.45-μm filter and degassed prior to use. Protein solutions were titrated by the addition of 20 × 14 μL aliquots of ligand solution at 25 °C and with 7-min intervals in a MicroCal (Northampton, MA) Omega titration calorimeter (Wiseman et al., 1989). The binding parameters K_a, $\Delta H°$, and n (stoichiometry) were obtained through nonlinear least squares fit of the observed reaction heat for each titration step. The Gibbs free energy change upon binding ($\Delta G°$) was calculated using the equation $\Delta G° = -RT \ln K_a$. Measurements were corrected for heats of dilution of ligand into buffer alone. Titrations of low MW heparin with bFGF in varying ionic strengths were performed as described above except that 100 mM NaCl in the dialysis and titration buffers was replaced with 300, 500, or 700 mM NaCl.

The experimental observable measured by the calorimeter is the difference in heat, $\Delta Q_{(i)}$ between the i and $i-1$ injections: $\Delta Q_{(i)} = Q_{(i)} - Q_{(i-1)}$. This reaction heat, observed for each ith step, for a simple association reaction, M + X ↔ MX, can be expressed as $\Delta Q_{(i)} = Q_{(i)} - Q_{(i-1)} = \Delta H[MX_{(i)} - MX_{(i-1)}]$, where M is the macromolecule in the calorimetric cell, X is the ligand in the syringe, MX is the product,[2] $MX_{(i)}$ is the amount of product present at each step, i, and ΔH is the molar enthalpy of binding. The $[MX_{(i)}]$ will be a function of the $[X]_{total}$, in the cell at step i, the $[M]_{total}$, and the association constant K_a. A mathematical model that relates the change in the reaction heat with changes in the $[X]_{total}$, $\Delta Q_{(i)}/\Delta[X]_{total}$, to the parameters ΔH, $[M]_{total}$, n (stoichiometry), and the association constant, K_a, was previously described (Wiseman et al., 1989; Connelly et al., 1990). This model was used to simulate the binding isotherms, and an iterative nonlinear least squares fitting program, ORIGIN (MicroCal), was used to fit the simulated binding isotherms to the experimental $\Delta Q_{(i)}$ by floating n, K_a, and $\Delta H°$ as fitting parameters in a standard Marquardt fashion for the minimization of the sum of the squared residuals, i.e., χ^2 (Wiseman et al., 1989).

Molecular Modeling of the Interaction between bFGF and a Pentasaccharide Heparin Analog. All model building was conducted with modules of BioSym's (San Diego, CA) INSIGHT II version 2.0 or 2.10 software. Docking and calculations of intermolecular interaction energy, E_{inter}, were carried out with the DOCKING module, while energy minimization and molecular dynamics simulations were run using the DISCOVER version 6.8 module. The simulations were performed on a Silicon Graphics ESX480 workstation.

A heparin-like pentasaccharide model was constructed using the formula $GlcNSO_3$-(6-OSO_3)-α(1→4)-IdoA-α(1→4)-$GlcNSO_3$-(2-OSO_3)-(6-OSO_3)-α(1→4)-GlcUA-(3-OSO_3)-α(1→4)-$GlcNSO_3$-(6-OSO_3)-OMe, where $GlcNSO_3$-(6-OSO_3) is D-glucosamine *N*-6-disulfate, IdoA is L-iduronic acid, GlcUA is D-glucuronic acid, and the sulfate substituents are numbered using the IUPAC convention for carbohydrates. This particular pentasaccharide derivative was chosen as a model for heparin binding to bFGF because it has been prepared using organic synthetic techniques and has been well characterized for its binding to antithrombin III (Petitou et al., 1991). Moreover, it was used by Grootenhuis and van Boeckel (1991) to construct a model of the binding interactions between heparin and antithrombin III.

The force fields and charges for the oligosaccharide atoms were assigned using the consistent valence force field (CVFF) library in INSIGHT II, and the partial charges for the atoms of the sulfate groups of the sugar were assigned using the MOPAC program, a semiempirical molecular orbital program, also available within INSIGHT II. An energy minimization of the pentasaccharide was then performed using the program DISCOVER before attempting to dock this ligand to bFGF.

RESULTS

Identification of Target Residues by X-ray Crystallography. The residues in contact with bound sulfate anions observed in X-ray crystal structures of bFGF (Figure 1) served as likely candidates to provide specific interactions with the sulfated polysaccharide, heparin (Eriksson et al., 1991; Zhang et al., 1991). As suggested by these authors, the ordered sulfate anions that are in contact with the side chains of N27, K119, R120, K125, and K129 might comprise part of the heparin binding site of bFGF. Ago et al. (1991) and Zhang et al. (1991) also suggested that K119, R120, K125, K129, and K135 might comprise a heparin binding site based on a calculated electrostatic potential of the solvent-accessible surface on bFGF. More recently, Rees and colleagues reported the X-ray crystal structure of the sulfated polysaccharide sucrose octasulfate (SOS) bound to aFGF and indicated that aFGF residues K112 and K118 (K119 and K125 are the analogous residues in bFGF) are important amino acids in the interaction of aFGF with SOS (Zhu et al., 1993).

Identification of Target Residues by Molecular Modeling of the Interaction between bFGF and a Pentasaccharide Heparin Analog. In order to further identify amino acids potentially involved in specific heparin interactions, the positions of sulfate anions in the crystal structure of bFGF (Zhang et al., 1991) (Figure 1) were used as a guide to construct a model interaction of a heparin pentasaccharide docked onto the surface of bFGF (Figure 2). Docking of a pentasaccharide heparin model (see Experimental Procedures) to bFGF was initiated by using three pentasaccharide sulfate anionic groups to make a three-point landing onto the three sulfate anions observed in the bFGF X-ray crystal structure (Zhang et al., 1991) to obtain the best superposition of the sulfate anions in both molecules. The three sulfate anionic groups of the pentasaccharide model chosen were the –6-OSO_3^- of monosaccharide 1, the –6-OSO_3^- of monosaccharide 3, and the –NSO_3^- of monosaccharide 5 at the reducing end of the oligosaccharide model. The original three bFGF bound sulfate anions were then removed, the docked complex was associated, and the initial ligand–protein complex was subjected to conjugate gradient energy minimization in which all atoms were allowed to move, including the 67 water molecules observed in the bFGF crystal structure (1082 heavy atoms). The minimization was continued until the rms energy gradient was less than 0.2 kcal/(mol·Å^2). Following energy

[2] The convention for isothermal titrating calorimetry is to call the reactant in the calorimetric cell the macromolecule, M, and the reactant in the syringe the ligand, X (Wiseman et al., 1989; Lin et al., 1991; Connelly et al., 1990).

FIGURE 1: Ribbon diagram of the C_α backbone of bFGF with bound sulfate anions taken from the X-ray crystal structure coordinates (Zhang et al., 1991). Amino acid residues proposed as important in heparin binding, on the basis of contact with sulfate anions as seen in the X-ray crystal structure of bFGF, are shown in orange with the side chains displayed in CPK (see text). The bound sulfate anions are also displayed in CPK and are colored by atom (sulfur = yellow, oxygen = red). Additional amino acid side chains proposed to be important in heparin binding on the basis of a model of heparin docked to bFGF (see Figure 2) are displayed in CPK and shown as white. The side chain of R44 is also displayed in CPK (green) and is approximately 10 Å from the nearest sulfate anion.

minimization, the heparin–bFGF complex was relaxed using a 5-ps molecular dynamics run at 300 K (no constraints), followed again by conjugate gradient energy minimization. The resulting complex is shown in Figure 2. Inspection revealed that the following 10 amino acid residues have side chains that make contacts with the docked pentasaccharide model, K26, N27, K119, R120, T121, Q123, K125, K129, Q134, and K135, making them suitable targets for site-directed mutagenesis. Further inspection revealed that another side chain, R81, was not directly interacting with the pentasaccharide but was bridged through an intervening water molecule to the $GlcNSO_3^-$ group of monosaccharide 5 at the "reducing end" (–OMe) of the ligand. These 11 amino acids were replaced individually or in various combinations with alanine to evaluate their energetic contribution to the bFGF–heparin interaction.

[NaCl]-Dependent Elution from a H.Seph. Affinity Column. Basic FGF site-directed mutants first were examined for differences in [NaCl]-dependent elution from a H.Seph. affinity column to obtain a semiquantitative comparison of changes in heparin affinity. Protein binding to H.Seph. and [NaCl]-dependent elution have been used to examine heparin–protein interactions in other systems (Berryman & Bensadoun, 1993; Hata et al., 1993). Wild-type bFGF eluted at 1.38 M NaCl under the conditions employed in this experiment. All of the single-site mutations implicated by structure and modeling efforts as potentially important for heparin binding revealed diminished affinity to H.Seph. compared to wild-type bFGF. The R120A/K125A double mutation and the K119A/R120A/K125A/K129A quadruple mutation resulted in even more dramatically reduced heparin binding as evidenced by elution at 0.46 and 0.26 M NaCl, respectively (Table 1).

Altered NaCl elution for bFGF site-directed mutants from H.Seph. was confirmed to be due to specific binding rather than nonspecific cationic interactions in two ways. First, a control mutant protein was constructed in which arginine at amino acid position 44 was replaced with alanine (R44A). R44 is near the putative heparin binding site (~10 Å from the nearest sulfate anion) but not thought to be involved in heparin binding according to our docked heparin model or X-ray crystal structure analyses (Figures 1 and 2). Replacement of this surface-exposed, positively charged, amino acid with alanine had no effect on NaCl elution from H.Seph. (Table 1). Second, the extent of nonspecific (strictly cation exchange) contributions to bFGF binding to H.Seph. was estimated by the interactions of trypsinogen with H.Seph. Trypsinogen has no known specific interaction with heparin, yet it binds to H.Seph. at neutral pH due to a basic p*I* (~9.3) and elutes at 0.22 M NaCl. Trypsinogen thus serves as a control to monitor the [NaCl] elution from H.Seph. due to nonspecific cation-exchange interactions. The isoelectric point of trypsinogen is similar to that of the bFGF K119A/R120A/K125A/K129A quadruple mutant (data not shown). Both the quadruple mutant and trypsinogen elute from H.Seph. at a similar [NaCl], suggesting that the K119A/R120A/K125A/K129A quadruple mutation eliminated most of the specific heparin binding interactions and that the observed binding to H.Seph. is due to the cation-exchange properties of the negatively charged H.Seph. affinity column. However, the more subtle differences in [NaCl] elution from H.Seph. observed for the single-site mutants can be considered to be due mostly to changes in specific heparin binding. This is supported by the observed correlation between [NaCl] elution from H.Seph. and the binding affinity measured by isothermal titration calorimetry (see Discussion, Figure 6).

Isothermal Titrating Calorimetry. The binding of low MW heparin to wild-type and mutant bFGF's was further characterized by isothermal titration calorimetry. The binding parameters n (stoichiometry), K_a, and $\Delta H°$ were derived from the nonlinear least squares fit of the binding isotherms (Wiseman et al., 1989). Representative titrations of low MW heparin with wild-type bFGF and the K125A mutant are presented in Figures 3 and 4, respectively. A summary of the observed and calculated thermodynamic terms for all of the mutants is presented in Table 1.

The enthalpically dominated binding of low MW heparin to wild-type bFGF was found to be characterized by a single binding constant of K_d = 0.47 μM when the oligosaccharide was titrated into solutions of bFGF. The stoichiometry was observed to be 0.48, indicating that two bFGF molecules bind per one molecule of low MW heparin (M_r ~3000) under conditions where bFGF is in excess. Importantly, when the order of the reactants was reversed, such that bFGF was titrated into solutions of low MW heparin, the K_d remained unchanged (K_d = 0.46 μM); however, the observed stoichiometry = 1.96, which is consistent with the original observation that two bFGF molecules bind per one molecule of low MW heparin when sufficient bFGF is added to saturate all available binding sites. A stoichiometry of two bFGF molecules per one molecule of low MW heparin (M_r ~ 3000) is also consistent with the docking simulations of the modeled pentasaccharide (M_r = 1728) binding to one molecule of bFGF (Figure 2). In addition, titrations of bFGF into solutions of RD Heparin 5000 (M_r ~5000) resulted in an increased stoichiometry (n = 3.14 ± 0.02, K_d = 1.3 ± 0.28 μM), again consistent with

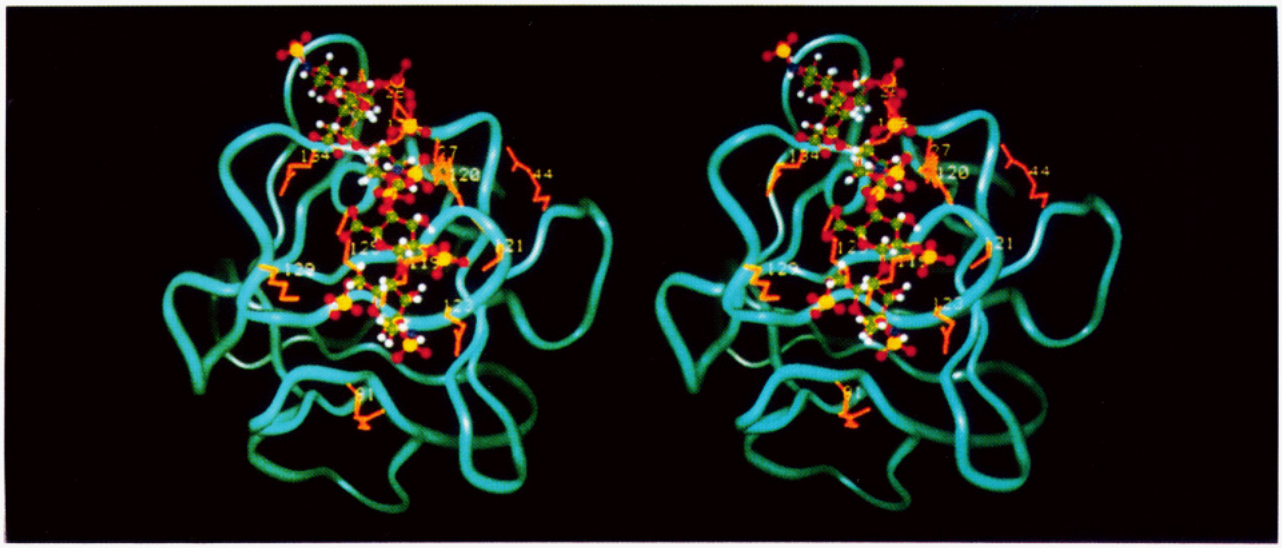

FIGURE 2: Stereo diagram of a molecular model of heparin pentasaccharide, $GlcNSO_3$-(6-OSO_3)-α(1→4)-IdoA-α(1→4)-$GlcNSO_3$-(2-OSO_3)-(6-OSO_3)-α(1→4)-GlcUA-(3-OSO_3)-α(1→4)-$GlcNSO_3$-(6-OSO_3)-OMe, docked to the surface of bFGF (see text for details). The bFGF side chains involved in heparin binding are shown in orange and numbered according to Zhang et al. (1991). Atoms of the pentasaccharide are colored by atom (sulfur = yellow; oxygen = red; nitrogen = blue; carbon = green; hydrogen = white). The resultant structure is after energy minimization of the initial docked complex, followed by 5-ps relaxation of molecular dynamics and by another round of energy minimization.

Table 1: Parameters for Binding Low MW Heparin to Wild-Type and Mutant bFGF's[a]

protein	H.Seph. elution[b] (M NaCl)	n	K_d (μM)	$\Delta H°$ [c] (kcal/mol)	$\Delta G°$ (kcal/mol)	$\Delta\Delta G°$ (kcal/mol)
wt bFGF[d]	1.38	0.48 ± 0.01	0.47 ± 0.01	−11.19 ± 0.51	−8.66	0.00
T121A	1.21	0.49 ± 0.01	0.69 ± 0.14	−17.47 ± 0.71	−8.43	0.24
R81A	1.20	0.57 ± 0.02	0.87 ± 0.04	−13.24 ± 0.92	−8.29	0.37
Q123A	1.16	0.48 ± 0.01	1.02 ± 0.25	−14.63 ± 0.10	−8.20	0.47
K26A	0.96	0.50 ± 0.01	1.15 ± 0.11	−12.25 ± 0.65	−8.13	0.54
K129A	0.99	0.47 ± 0.01	1.46 ± 0.42	−13.61 ± 0.17	−7.99	0.68
K135A	1.03	0.51 ± 0.04	1.52 ± 0.19	−12.25 ± 0.81	−7.96	0.70
K119A	1.02	0.55 ± 0.06	1.67 ± 0.25	−14.14 ± 1.28	−7.90	0.76
R120A	0.99	0.64 ± 0.05	2.54 ± 0.49	−14.37 ± 1.18	−7.66	1.01
Q134A	0.83	0.61 ± 0.01	2.73 ± 0.05	−13.70 ± 0.50	−7.61	1.05
N27A	0.84	0.54 ± 0.02	2.93 ± 0.46	−11.30 ± 0.09	−7.57	1.09
K125A	0.63	0.71 ± 0.02	7.89 ± 1.51	−9.76 ± 0.62	−6.98	1.68
R120A/K125A	0.43	0.70 ± 0.12	17.11 ± 3.76	−6.90 ± 0.26	−6.52	2.14
K119A/R120A/K125A/K129A[e]	0.26		(>28)[f]			
R44A	1.42					
Q123R	1.38					

[a] Titration calorimetry experiments were performed in 50 mM Hepes, 100 mM NaCl, and 1 mM DTT, pH 7.5, at 25 °C. The data were analyzed by assuming two noninteracting sites on the low MW heparin ($M_r \sim 3000$). The fitting parameters of K_d, $\Delta H°$, and n were obtained through a nonlinear least squares fit of the reaction heat observed for each ith step of a titration experiment for a simple association reaction (see text). Data are reported as the mean for at least three separate titrations with ±1 SD. [b] Values are reported with an estimated error of ±10%. [c] Enthalpies are reported for binding 2 bFGF/mol of low MW heparin. [d] When the order of addition of the reactants was reversed so that bFGF was titrated into solutions of low MW heparin, K_d was unchanged, but $n = 1.96$. [e] Due to precipitation of protein in the presence of heparin, a K_d could not be experimentally determined by titration calorimetry. [f] Estimated from Figure 6.

an FGF binding site on heparin of between four and five monosaccharides (~25 Å in length). Mach et al. (1993) reported a similar number of interacting monosaccharides per molecule of FGF for binding a larger heparin (M_r ~16 000) and also RD Heparin 5000 (M_r ~5000) to aFGF.

All titrations were performed using the same lot of low MW heparin to ensure uniformity. Commercially available heparins are typically inhomogeneous with respect to the degree of sulfation and molecular weight owing to the variable synthesis in cells and the method of depolymerization by commercial sources [for recent reviews see Gallagher and Turnbull (1992) and Yanagishita and Hascall (1992)]. The low MW heparin sample (M_r ~3000) and the RD Heparin 5000 (M_r ~5000) are from two different commercial sources and were prepared by two different depolymerization methods, peroxidolysis and nitrous acid, respectively. Each derivative was found to bind to bFGF with very similar binding parameters, suggesting that the microheterogeneity within each sample is undetectable under these experimental conditions. The absence of bi- or multicomponent binding isotherms, and the indifference to the method of preparation or commercial source of the two heparin derivatives, suggests that the binding of low MW heparin to bFGF can be accurately represented by the binding constants reported in Table 1.

The apparent K_d values of binding low MW heparin for each of the single-site mutants and one double mutant (R120A/K125A) are also reported in Table 1. The most dramatic change in K_d for any of the single-site mutants was observed for K125A, in which the binding affinity for heparin decreased 17-fold relative to wild-type bFGF. The calculated $\Delta G°$ for wild-type bFGF = −8.7 kcal/mol. The $\Delta G°$ for K125A = −7.0 kcal/mol, resulting in a $\Delta\Delta G°$ for K125A = 1.7 kcal/mol. Therefore, K125 alone provides approximately 20% of the free energy of bFGF binding to low MW heparin.

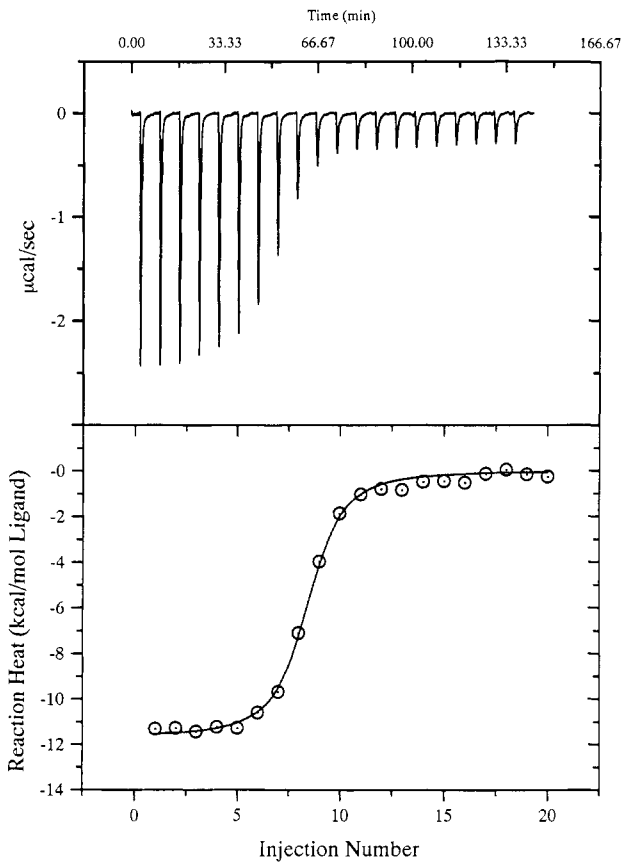

FIGURE 3: Representative binding isotherm for the titration of low MW heparin (M_r ~3000) into solutions of wild-type bFGF. A 430 μM solution of low MW heparin was titrated into a 84 μM bFGF solution using a 20 × 14 μL injection schedule spaced at 7-min intervals (T = 24.9 °C). The rate of this reaction was sufficiently fast under these conditions (k_{on} = 0.9 × 10^8 M^{-1} min^{-1} for bFGF binding to HSPG; Nugent & Edelman, 1992) that this injection schedule allowed complete equilibration between additions. The bFGF solution had been dialyzed vs 2 × 1 L of 50 mM Hepes, 0.10 M NaCl, and 1.0 mM DTT, and the heparin solution was prepared by dissolving solid low MW heparin in the same buffer. (Top panel) Raw titration data for wild-type bFGF showing the 20 individual changes in $Q_{(i)}$ after each injection of heparin. (Bottom panel) Resultant binding isotherm for low MW heparin titration of bFGF after integration of the area under each injection peak and subtraction of the blank. The solid line represents a nonlinear least squares fit of the reaction heat for each injection ($\Delta Q_{(i)}$) with the assumption of a single binding site comprised of the three fitting parameters, n, K_a, and $\Delta H°$, which were all allowed to "float" during computer iterations. This experiment was carried out at least three times for each protein, and a summary of the binding parameters is presented in Table 1.

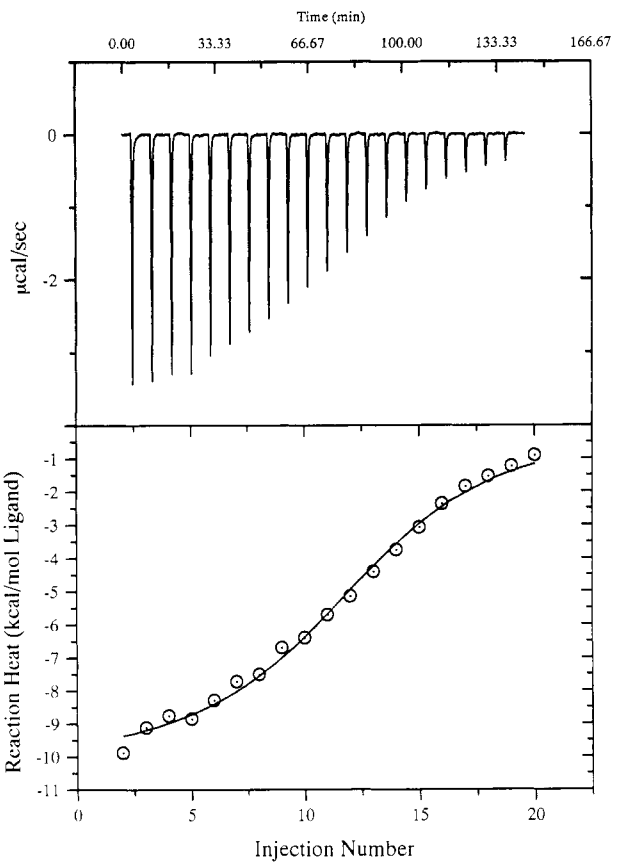

FIGURE 4: Representative binding isotherm for the titration of low MW heparin into solutions of bFGF mutant K125A. A 153 μM solution of K125A bFGF was equilibrated in the calorimetric cell and titrated with 20 × 14 μL injections (14 μL delivered over a 12-s duration) of 765 μM low MW heparin (M_r ~3000) by employing an injection schedule that separated the additions by 7 min. The top panel is the raw data and the bottom panel is the resultant binding isotherm for low MW heparin titration of K125A after integration of the area under each injection peak and subtraction of the blank. The solid line represents a nonlinear least squares fit of the reaction heat for each injection, $\Delta Q_{(i)}$, after subtraction of the blank.

Amino acids which contribute at least 1 kcal/mol to the free energy of binding include N27, R120, K125, and Q134. The sum of the individual $\Delta\Delta G°$ values for all of the single-site mutants = 8.6 kcal/mol, in close agreement with the observed $\Delta G°$ = –8.7 kcal/mol of binding for wild-type bFGF. Therefore, within the experimental error of this system, we can account for >95% of the binding free energy of a low MW heparin binding to bFGF through the interactions of the amino acids identified above as comprising the heparin binding site. The $\Delta\Delta G°$ for the double mutant R120A/K125A = 2.1 kcal/mol. As expected, the effect of simultaneously replacing two amino acids involved in heparin binding increases the apparent K_d more than either single mutant alone, and the $\Delta\Delta G°$ for R120A/K125A approaches the sum of the $\Delta\Delta G°$ for R120A + K125A ($\Delta\Delta G°$ = 2.7 kcal/mol).

Ionic Strength Dependence of Heparin Binding to bFGF. Titration of low MW heparin to wild-type bFGF in differing [NaCl] was undertaken to differentiate the ionic vs nonionic components of bFGF binding to heparin. The dependence of ionic strength on binding was analyzed according to the theory of macromolecule–polyelectrolyte interactions (Record et al., 1976, 1978; Manning, 1978) with the assumption that heparin behaves as a polyelectrolyte in solution (Olson et al., 1991; Faller et al., 1992). The theory predicts that binding of a protein (bFGF) to a polyelectrolyte (heparin) in the presence of a counterion (Na^+) will result in the stoichiometric release of Na^+ from the heparin polymer. At equilibrium the process may be described as follows:

$$\text{bFGF}^{Z+} + \text{heparin} \overset{K_d}{\leftrightarrow} \text{bFGF–heparin} + Z\psi\text{Na}^+ \quad (1)$$

where Z is the number of purely ionic interactions formed between bFGF and heparin which result in the displacement of $Z\Psi$-bound Na^+ from heparin and Ψ represents the fraction of Na^+ ions bound to heparin and is dependent on the axial charge density of the polymer (Record et al., 1976; Diakun et al., 1978). A value of Ψ = 0.8 has been reported for heparin (Mattai & Kwak, 1981; Olson et al., 1991; Faller et al., 1992). Therefore, the observed K_d is related to the K_d(nonionic) as follows:

$$\log K_d = \log K_d(\text{nonionic}) + Z\psi \log [\text{Na}^+] \quad (2)$$

A plot of log K_d vs log [Na^+] is linear, and the slope (=1.95)

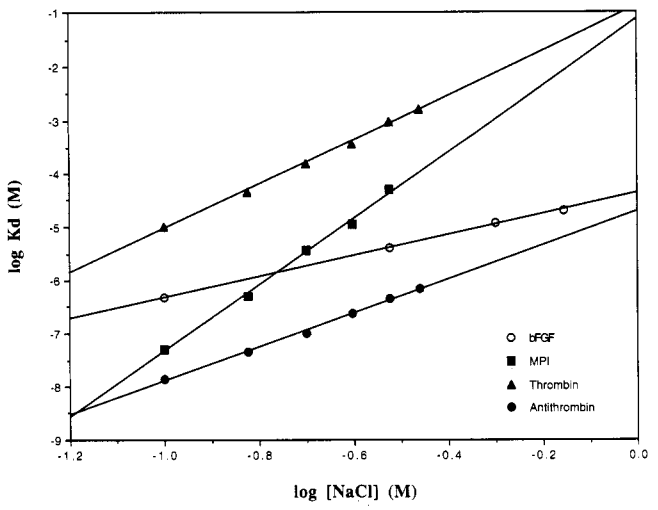

FIGURE 5: Ionic strength dependence for low MW heparin binding to bFGF. The log K_d values were plotted as a function of the log [NaCl]. Data shown are for heparin with MPI (■) (Faller et al., 1992), heparin with thrombin (▲) or antithrombin (●) (Olson et al., 1991; Olson & Björk, 1991), and heparin with bFGF (○) (this paper). The heparin–bFGF interaction was measured at pH 7.5 and 25 °C in 50 mM Hepes buffer. For bFGF–heparin binding K_d = 0.47 ● 0.01 μM, 4.10 ± 0.50 μM, 11.80 ± 1.50 μM, and 21.25 ± 7.15 μM for [NaCl] = 0.1, 0.3, 0.5, and 0.7 M, respectively; errors are ●1 SD. Error bars are smaller than legend symbols and are not shown.

provides an estimate of the purely ionic interactions involved in the binding of bFGF to low MW heparin (Figure 5). Using Ψ = 0.8, the binding of heparin to bFGF displaces two to three Na^+ ions. This suggests that between two and three net purely ionic interactions are involved in the bFGF–low MW heparin complex. At 1.0 M NaCl all ionic interactions can be considered neutralized, and log K_d = log K_d(nonionic). Therefore, the Y intercept of the plot of log K_d vs log [NaCl] = K_d(nonionic) = 42 μM, whereas the observed apparent K_d for bFGF–heparin binding = 470 nM at 0.1 M NaCl. Comparison of log K_d(nonionic) (–4.4) vs observed log K_d (–6.3) reveals that only ~30% of the binding free energy is derived from purely ionic interactions at pH 7.5 and 25 °C. This result is similar to that observed for the interaction of heparin with antithrombin (Olson & Björk, 1991), yet considerably different than that observed for the binding of heparin to thrombin or mucus proteinase inhibitor (Olson et al., 1991; Faller et al., 1992) (Figure 5, see Discussion).

DISCUSSION

Published X-ray crystal structures of bFGF (Eriksson et al., 1991; Zhang et al., 1991) implicated amino acids N27, K119, R120, K125, K129, and K135 as potential contributors to heparin binding, although the relative contribution of these residues to binding was uncertain. The molecular modeling efforts of a pentasaccharide form of heparin docked to bFGF tentatively confirmed these residues as components of the heparin binding interaction and also suggested K26, R81, T121, Q123, and Q134 as additional candidates (see Results). These 11 putative heparin binding amino acids were replaced individually with alanine, and mutant proteins were initially characterized according to their relative [NaCl]-dependent elution from H.Seph. The single-site mutants N27A, Q134A, and K125A were the most compromised in heparin affinity. The basic FGF mutants T121A, Q123A, and R81A were the least altered, with the remaining mutants revealing intermediate H.Seph. [NaCl]-dependent elution (Table 1). More quantitative determinations of mutant bFGF's binding to low MW heparin were provided by isothermal titration calorimetry (Table 1). From $\Delta\Delta G°$ values, K125 alone is responsible for ~20% of the total binding energy, indicating that this residue is the single most important bFGF residue involved in heparin binding. This result is consistent with the work of Harper and Lobb (1988) and Burgess et al. (1990) in which the analogous lysine residue in aFGF (K118) was chemically modified or replaced with glutamic acid by site-directed mutagenesis, respectively, and thus determined to be important in heparin binding.

Characterization of bFGF deletion mutants for differences in heparin binding, based on [NaCl] elution from a H.Seph. affinity column, had previously implicated residues in the C-terminal domain as important for binding heparin (Seno et al., 1990). Our results confirm these earlier findings and provide a detailed quantitation of the relative contributions of each amino acid. The sum $\Delta\Delta G°$ = 6.6 kcal/mol for the C-terminal residues between amino acid numbers 119 and 135 that were subjected to replacement with alanine (Table 1). Although the heparin binding domain is discontinuous, the linear sequence in bFGF from amino acid number 119 to 135 accounts for ~76% of the binding free energy. On the basis of the results from H.Seph. binding and titration calorimetry, all of the bFGF amino acids predicted by structure and modeling efforts to be important in heparin binding appear to contribute to the specific heparin–bFGF interaction.

A series of titrations of wild-type bFGF binding to low MW heparin were conducted in increasing [NaCl] to quantitate ionic versus nonionic contributions to the binding energy (Figure 5). Due to the combination of clustered positive residues on bFGF and the overall negative charge on heparin, it was anticipated that the binding reaction would be dominated by electrostatic interactions. In addition, the crystal structures of bFGF indicated that R120 and K125 were in direct contact with sulfate anions. The mutagenesis experiments, however, revealed that two uncharged amino acids, N27 and Q134, provide a significant contribution (~25%) to the overall binding energy (Table 1). The bFGF with docked heparin model suggested that Q134 was in a position to interact with the polysaccharide, and the X-ray crystal structures of bFGF suggested that N27 was in direct contact with a sulfate anion (Figure 2). These two residues, along with Q123 and T121, are the only uncharged amino acids identified in the heparin binding domain, and together they account for ~33% of the total binding free energy.

In contrast to the interaction of heparin with thrombin or heparin with mucus proteinase inhibitor (MPI) in which >80% of the binding energy was determined to be due to ionic interactions, the bFGF–heparin binding energy is dominated by nonionic interactions (Figure 5). A 3-fold increase in [NaCl] (0.1–0.3 M NaCl) decreased the binding affinity of heparin to MPI by 1000-fold (50 nM vs 50 μM), whereas a 7-fold increase in [NaCl] (0.1–0.7 M NaCl) decreased the binding affinity of low MW heparin to bFGF by less than 40-fold (0.47 vs 17.2 μM). MPI and thrombin are estimated to have at least seven and five net ionic interactions in heparin binding, respectively (Figure 5; Olson et al., 1991; Faller et al., 1992). We estimate that only two to three net ionic interactions are involved in the bFGF–heparin complex and that approximately 30% of the binding energy results from purely ionic interactions. In this regard, the ionic strength dependence of the bFGF–heparin complex is analogous to the binding of heparin to antithrombin in which it can be estimated that 60% of the binding energy is due to nonionic interactions (Figure 5; Olson & Björk, 1991). The observation that bFGF binding to low MW heparin is dominated by nonionic

interactions is consistent with the requirement of >1 M NaCl to elute wild-type bFGF from a H.Seph. affinity column, whereas MPI elutes at less than 1 M NaCl even though the K_d for the MPI–heparin complex is 50 nM (Faller et al., 1992) compared to 470 nM for low MW heparin binding to bFGF (Table 1).

On the basis of the observation that ~33% of the binding free energy of bFGF binding to low MW heparin is derived from the nonionic interactions of amino acids N27, T121, Q123, and Q134 and that only ~30% of the binding free energy is derived from purely electrostatic interactions, the remaining binding free energy (~37%) must be due to hydrogen bonding, van der Waals packing, and hydrophobic interactions contributed by charged amino acid side chains. For example, the cationic N_ζ of K125 was found to be 2.8 Å from the 6-OSO_3^- group of monosaccharide residue 3, GlcNSO$_3$-(2-OSO_3^-)-(6-OSO_3^-), and 2.7 Å from a carboxylate O atom of the GlcUA-(3-OSO_3^-) monosaccharide residue 4 in the docked ligand complex (Figure 2). These distances are ideal for the electrostatic component of molecular interactions but are also optimum distances for hydrogen-bonding interactions. Another observation that is apparent in Figure 2 concerns the partial burial of the K125 side chain upon binding low MW heparin, implying that a hydrophobic/van der Waals packing component derived from the aliphatic portion of this side chain might also contribute to the binding free energy. These observations are consistent with the report that upon reductive methylation of aFGF residue K118 (analogous to K125 of bFGF) the side chain retains a net positive charge, yet binding to heparin is decreased substantially (Harper & Lobb, 1988). Therefore, the 1.7 kcal/mol of binding energy contributed by the K125 amino acid side chain most likely reflects a combination of electrostatic (ionic), hydrogen-bonding (nonionic), and hydrophobic/van der Waals packing (nonionic) components. Many of the other cationic residues of bFGF that are in contact with the modeled heparin pentasaccharide were also found to be within hydrogen-bonding distance to hydrogen bond acceptors on the ligand. The observed experimental $\Delta\Delta G°$ for the other lysine and arginine mutants (Table 1) are also likely to reflect multicomponent energy contributions from ionic and nonionic sources. The importance of specific hydrogen bond formation and/or van der Waals packing in oligosaccharide recognition and discrimination is further evidenced by the observation that chondroitin sulfate, another highly sulfated polysaccharide, does not bind to bFGF (Gospodarowicz & Cheng, 1986; Roghani & Moscatelli, 1992).

A comparison of heparin binding by titration calorimetry and by NaCl elution from H.Seph. is presented in Figure 6. Increasing apparent K_d values measured by isothermal titration calorimetry for site-directed mutants correlate well with a decreasing [NaCl] required to elute the same mutant proteins from H.Seph. This correlation has proved to be a rapid and accurate method to predict the K_d of bFGF mutants once the elution profile from H.Seph. has been established. This comparison using bFGF mutants lends credence to the use of NaCl elution from a H.Seph. affinity column as a reliable means of determining relative differences in heparin binding and may be applicable to other heparin binding proteins as well.

A sequence alignment of members of the FGF family revealed a high degree of conservation among the various heparin binding FGF polypeptides for the amino acids reported in this paper as important in heparin binding (data not shown). The conservation of key heparin binding residues is particularly

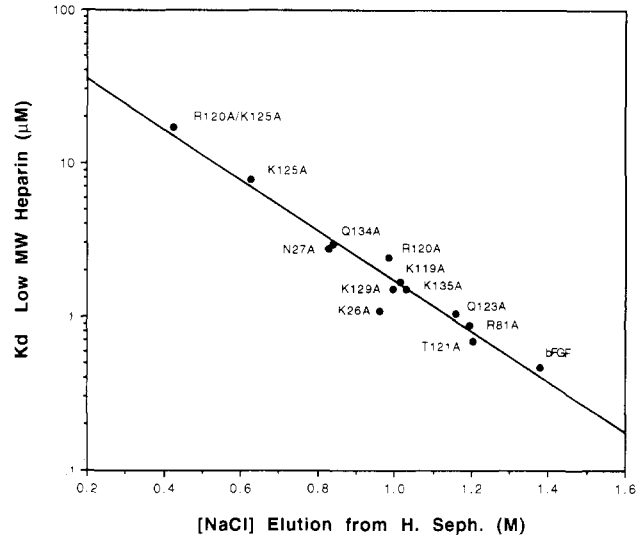

FIGURE 6: Correlation of observed K_d's for wild-type and mutant bFGF's binding to low MW heparin determined by titrating calorimetry vs [NaCl] elution from a heparin–Sepharose affinity column. Values were taken from Table 1.

good with aFGF. The only residues not conserved are at bFGF amino acid positions K26 and R81 in which aFGF presents serine and leucine, respectively. On the basis of the sum $\Delta\Delta G°$ value for bFGF mutants K26A and R81A, these two residues combined contribute less than 1 kcal/mol to the total binding. Molecular modeling efforts suggested that one possible compensating change might be the presence of arginine in aFGF rather than glutamine at bFGF position 123. Replacement of Q123 with arginine in bFGF by site-directed mutagenesis resulted in no change in binding affinity (Table 1) rather than a predicted enhancement, suggesting that the arginine side chain at this position in bFGF has no advantage over glutamine. Acidic FGF elutes from H.Seph. at a lower [NaCl] than bFGF (Harper & Lobb, 1988), suggesting a lower binding affinity to heparin than bFGF. The sequence differences between aFGF and bFGF mentioned above might explain the differences in heparin affinity. On the basis of the high degree of conservation in the heparin binding domain of bFGF and aFGF, we predict that the heparin binding site on aFGF will be comprised largely of the analogous residues determined to be important in bFGF. Amino acids in the other FGF's found at analogous positions to the residues identified in bFGF as important for heparin binding will most likely comprise the heparin binding domains of these growth factors as well.

Addition of exogenous heparin in cell assay systems which lack HSPG seems required for cell proliferation, thereby implicating heparin as a critical element in FGF-induced mitogenicity (Rapraeger et al., 1991; Yayon et al., 1991; Ornitz et al., 1992). The titrating calorimetry experiments reported above yield stoichiometries that are consistent with two molecules of bFGF binding to one molecule of low MW heparin (under conditions where heparin is limiting), with each binding unit being comprised of five sugar units. Ishihara et al. (1992) and Tyrrell et al. (1993) have examined the ability of affinity-purified, heparin-derived oligosaccharides to bind to endothelial cells and to promote proliferation. Their experiments indicate that hexasaccharides are the smallest heparin-derived polysaccharides that are capable of binding to bFGF with high affinity and that the smallest biologically active oligosaccharide contains a minimum of 10 monosaccharides. Similar experiments have found an octamer to be the smallest biologically active heparin (Ornitz et al., 1992). The smallest

oligosaccharide that can bind tightly to bFGF appears to be approximately five or six monosaccharides in length, yet an additional four to six monosaccharides are required for bFGF to elicit a biological response.

One possible explanation for the requirement of heparin binding in bFGF-mediated mitogenicity might be to provide a template for binding multiple bFGF molecules, thus increasing the local concentration of the growth factor. This may in turn promote FGF receptor dimerization and subsequent signal transduction upon binding bFGF (Ornitz et al., 1992; Mach et al., 1993). As the stoichiometry data in Table 1 indicate, two FGF molecules can bind per heparin $M_r \sim 3000$. Thus, an oligosaccharide of 8–12 monosaccharides would be sufficient to allow binding of two FGF's and thereby bring two FGFR's close enough to allow dimerization. Rees and co-workers suggested a similar possible mechanism based on the X-ray crystal structure of sucrose octasulfate bound to bFGF (Zhu et al., 1993).

A second possible role for heparin centers on the recent observation that FGFR also binds heparin in a specific manner (Kan et al., 1993; Pantoliano et al., 1994). Pantoliano and co-workers have dissected the individual binary binding reactions between FGF with FGFR, FGF with heparin, and FGFR with heparin and implicated the energetic coupling of these interactions in the assembly of higher order macromolecular complexes. In this scenario, heparin binding could juxtapose the growth factor and receptor for efficient binding and possibly invoke a second receptor binding site on FGF to permit receptor dimerization in a mechanism similar to that observed for human growth hormone and its receptor (Cunningham et al., 1991; De Vos et al., 1992). Specific heparin binding to FGFR adds another level of complexity to the FGF system that will require further investigation before the biological significance of coupled heparin–bFGF–FGFR interactions is completely understood.

ACKNOWLEDGMENTS

The authors thank Rob Horlick and Will Sisk for providing the bFGF clone and expression construct, Stephen Sprang and Doug Rees for the bFGF and aFGF X-ray coordinates, Patricia Weber and Michael Levitt for helpful discussions, Stuart Rosenfeld and Harold Ross for providing cell paste for some of the mutants, Marvin Kendall for synthesized oligonucleotides, Anil Dwivedi for the circular dichroism spectra, and the DuPont Merck postdoctoral program.

REFERENCES

Ago, H., Kitagawa, Y., Fujishima, A., Matsuura, Y., & Katsube, Y. (1991) *J. Biochem. 110,* 360–363.

Baird, A., Schubert, D., Ling, N., & Guillemin, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 2324–2328.

Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C. M., Folkman, J., & Vlodavsky, I. (1989) *Biochemistry 28,* 1737–1743.

Berryman, D. E., & Bensadoun, A. (1993) *J. Biol. Chem. 268,* 3272–3276.

Burgess, W. H., & Maciag, T. (1989) *Annu. Rev. Biochem. 58,* 575–606.

Burgess, W. H., Shaheen, A. M., Ravera, M., Jaye, M., Donohoe, P. J., & Winkles, J. A. (1990) *J. Cell Biol. 111,* 2129–2138.

Connelly, P. R., Varadarajan, R., Sturtevant, J. M., & Richards, F. M. (1990) *Biochemistry 29,* 6108–6114.

Cunningham, B. C., Ultsch, M., De Vos, A. M., Mulkerrin, M. G., Clauser, K. R., & Wells, J. A. (1991) *Science 254,* 821–825.

Davidson, J. M., Klagsbrun, M., Hill, K. E., Buckley, A., Sullivan, R., Brewer, P. S., & Woodward, S. C. (1985) *J. Cell Biol. 100,* 1219–1227.

De Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) *Science 255,* 306–312.

Diakun, G. P., Edwards, H. E., Wedlock, D. J., Allen, J. C., & Phillips, G. O. (1978) *Macromolecules 11,* 1110–1114.

Eriksson, A. E., Cousens, L. S., Weaver, L. H., & Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 3441–3445.

Faller, B., Mely, Y., Gerard, D., & Bieth, J. G. (1992) *Biochemistry 31,* 8285–8290.

Flaumenhaft, R., Moscatelli, D., & Rifkin, D. B. (1990) *J. Cell Biol. 111,* 1651–1659.

Folkman, J., & Klagsbrun, M. (1987) *Science 235,* 442–447.

Gallagher, J. T., & Turnbull, J. E. (1992) *Glycobiology 2,* 523–528.

Gospodarowicz, D. (1987) *Methods Enzymol. 147,* 106–119.

Gospodarowicz, D., & Cheng, J. (1986) *J. Cell. Physiol. 128,* 475–484.

Gospodarowicz, D., Cheng, J., Lui, G.-M., Baird, A., & Böhlent, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81,* 6963–6967.

Grootenhuis, P. D. J., & van Boeckel, C. A. A. (1991) *J. Am. Chem. Soc. 113,* 2743–2747.

Harper, J. W., & Lobb, R. R. (1988) *Biochemistry 27,* 671–678.

Hata, A., Ridinger, D. N., Sutherland, S., Emi, M., Shuhua, Z., Myers, R. L., Ren, K., Cheng, T., Inoue, I., Wilson, D. E., Iverius, P.-H., & Lalouel, J.-M. (1993) *J. Biol. Chem. 268,* 8447–8457.

Heath, W. F., Cantrell, A. S., Mayne, N. G., & Jaskunas, S. R. (1991) *Biochemistry 30,* 5608–5617.

Ishihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S., Cousens, L. J., & Stack, R. J. (1993) *J. Biol. Chem. 268,* 4675–4683.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., & McKeehan, W. L. (1993) *Science 259,* 1918–1921.

Kiefer, M. C., Baird, A., Nguyen, T., George-Nascimento, C., Mason, O. B., Boley, L. J., Valenzuela, P., & Barr, P. J. (1991) *Growth Factors 5,* 115–127.

Klagsbrun, M., & Shing, Y. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82,* 805–809.

Klagsbrun, M., & Baird, A. (1991) *Cell 67,* 229–231.

Lin, L.-N., Mason, A. B., Woodworth, R. C., & Brandts, J. F. (1991) *Biochemistry 30,* 11660–11669.

Mach, H., Volkin, D. B., Burke, C. J., Middaugh, C. R., Linhardt, R. J., Fromm, J. R., Loganathan, D., & Mattsson, L. (1993) *Biochemistry 32,* 5480–5489.

Manning, G. S. (1978) *Q. Rev. Biophys. 11,* 179–246.

Mattai, J., & Kwak, J. C. T. (1981) *Biochim. Biophys. Acta 667,* 303–312.

McGee, G. S., Davidson, J. M., Buckley, A., Sommer, A., Woodward, S. C., Aquino, A. M., Barbour, R., & Demetriou, A. A. (1988) *J. Surg. Res. 45,* 145–153.

Moscatelli, D. (1987) *J. Cell. Physiol. 131,* 123–130.

Moscatelli, D. (1988) *J. Cell Biol. 107,* 753–759.

Moses, M. A., & Langer, R. (1991) *Bio/Technology 9,* 630–634.

Nabel, E. G., Yang, Z-y., Plautz, G., Forough, R., Zhan, X., Haudenschild, C. C., Maciag, T., & Nabel, G. J. (1993) *Nature 362,* 844–846.

Neufeld, G., & Gospodarowicz, D. (1985) *J. Biol. Chem. 260,* 13860–13868.

Nugent, M. A., & Edelman, E. R. (1992) *Biochemistry 31,* 8876–8883.

O'Keefe, E. J ., Chiu, M. L., & Payner, R. E., Jr. (1988) *J. Invest. Dermatol. 90,* 767–769.

Olson, S. T., & Björk, I. (1991) *J. Biol. Chem. 266,* 6353–6364.

Olson, S. T., Halvorson, H. R., & Björk, I. (1991) *J. Biol. Chem. 266,* 6342–6352.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., & Leder, P. (1992) *Mol. Cell. Biol. 12,* 240–247.

Pantoliano, M. W., Horlick, R. A., Van Dyk, D. E., Tobery, T., Duke, J. L., Nahapetian, A. T., Herblin, W. F., Springer, B. A., Bradley, J. D., & Sisk, W. P. (1994) *Biochemistry* (in press).

Petitou, M., Lormeau, J.-C., & Choay, J. (1991) *Nature 350* (Suppl.), 30–33.

Presta, M., Statuto, M., Isacchi, A., Caccia, P., Pozzi, A., Gualandris, A., Rusnati, M., Bergonzoni, L., & Sarmientos, P. (1992) *Biochem. Biophys. Res. Commun. 185*, 1098–1107.

Prestrelski, S. J., Fox, G. M., & Arakawa, T. (1992) *Arch. Biochem. Biophys. 293*, 314–319.

Rapraeger, A. C., Krufka, A., & Olwin, B. B. (1991) *Science 252*, 1705–1708.

Record, M. T., Jr., Lohman, T. M., & deHaseth, P. (1976) *J. Mol. Biol. 107*, 145–158.

Record, M. T., Jr., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.

Roghani, M., & Moscatelli, D. (1992) *J. Biol. Chem. 267*, 22156–22162.

Saksela, O., Moscatelli, D., Sommer, A., & Rifkin, D. B. (1988) *J. Cell Biol. 107*, 743–751.

Saunders, S., Jalkanen, M., O'Farrell, S., & Bernfeld, M. (1989) *J. Cell Biol. 108*, 1547–1556.

Seno, M., Sasada, R., Kurokawa, T., & Igarashi, K. (1990) *Eur. J. Biochem. 188*, 239–245.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., & Klagsbrun, M. (1984) *Science 223*, 1296–1299.

Shipley, G. D., Keeble, W. W., Hendrickson, J. E., Coffey, R. J., Jr., & Pittelkow, M. R. (1989) *J. Cell. Physiol. 138*, 511–518.

Sommer, A., & Rifkin, D. B. (1989) *J. Cell. Physiol. 138*, 215–220.

Squires, C. H., Childs, J., Eisenberg, S. P., Polverini, P. J., & Sommer, A. (1988) *J. Biol. Chem. 263*, 16297–16302.

Thompson, S. A., Protter, A. A., Bitting, L., Fiddes, J. C., & Abraham, J. A. (1991) *Methods Enzymol. 198*, 96–116.

Tyrrell, D. J., Ishihara, M., Rao, N., Horne, A., Kiefer, M. C., Stauber, G. B., Lam, L. H., & Stack, R. J. (1993) *J. Biol. Chem. 268*, 4684–4689.

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., & Klagsbrun, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2292–2296.

Wiseman, T., Williston, S., Brandts, J. F., & Lin, L.-N. (1989) *Anal. Biochem. 179*, 131–137.

Yanagishita, M., & Hascall, V. C. (1992) *J. Biol. Chem. 267*, 9451–9454.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841–848.

Zhang, J., Cousens, L. S., Barr, P. J., & Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3446–3450.

Zhu, X., Hsu, B. T., & Rees, D. C. (1993) *Structure 1*, 27–34.